Exhaled particles for monitoring of airway inflammation

Sampling and analysis of endogenous particles from breath

Per Larsson

Department of Public Health and Community Medicine
Institute of Medicine
Sahlgrenska Academy at University of Gothenburg

UNIVERSITY OF GOTHENBURG

Gothenburg 2016

Cover illustration: Schematic illustration of the instrument used for sampling exhaled particles. Illustration created by Per Larsson

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per.larsson@amm.gu.se

ISBN 978-91-628-9961-5 (Print)
ISBN 978-91-628-9962-2 (PDF)
Electronic version available at: http://hdl.handle.net/2077/44869

Printed in Gothenburg, Sweden 2016
Printed by Ineko AB

Dedicated to Meta and Algot who are always there to give me support

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Per Larsson

Department of Public Health and Community Medicine
Institute of Medicine
Sahlgrenska Academy at University of Gothenburg
Göteborg, Sweden

ABSTRACT

Non-invasive collection methods suitable for studying the composition of the respiratory tract lining fluid (RTLF) in small airways are currently not readily available. The overall aim of this thesis was to contribute to the development of a non-invasive method for sampling of RTLF with the purpose of studying airway inflammation.

As we breathe small particles of liquid are formed from the RTLF. These particles follow the airstream during exhalation and can be sampled from the exhaled breath using the PExA method. A micro sample of RTLF can thus be obtained by sampling these particles.

Methods for measuring surfactant protein A(SP-A) and albumin in exhaled particles (PEx) were developed. The methods were used to study the effect of birch pollen exposure in a group of individuals with mild asthma and birch pollen allergy. During birch pollen season the PEx mass concentration was reduced while no significant effect on SP-A or albumin concentrations in PEx was observed. Alteration in particle amount seem to reflect change in bronchial motor-tone. In a middle aged population without lung diseases, reference intervals (RI) for SP-A and albumin weight percent concentration (wt%) in PEx was calculated to 1.9-5.3 wt% and 3.6-11.2 wt% (90% RI), respectively. SP-A concentration in PEx was not associated with age, gender, anthropometry, atopy or particle production, whereas albumin concentration in PEx was associated to age, atopy and particle production.

Particle formation was studied with aim to understand and facilitate optimal particle collection. Particles exhaled with a maximal forced exhalation contained very low amounts of the major surfactant lipid (dipalmitoylphosphatidylcholine) compared to particles exhaled by a slow and deep exhalation/inhalation manoeuvre. This suggests that particle formation, including efficiency and formation site, can be controlled by selecting an appropriate breathing manoeuvre.

The PExA method is a promising non-invasive method for measuring proteins and lipids in RTLF collected from small airways. Further biomarker development studies are necessary to facilitate the method application in clinical studies.

Keywords: Exhaled particles, respiratory tract lining fluid, inflammation

ISBN: 978-91-628-9961-5 (Print)

SAMMANFATTNING PÅ SVENSKA

Lungsjukdom är en av de vanligaste dödsorsakerna i världen. När det gäller astma förekommer denna sjukdom främst i västvärlden, ca 6-8 % av den svenska befolkningen uppskattas ha någon form av astma.

Astma diagnostiseras ofta utifrån de symptom som sjukdomen orsakar, men de bakomliggande mekanismerna kan variera. Astma har traditionellt beskrivits som en inflammatorisk sjukdom men sambandet mellan inflammation och astma är inte klarlagt. Eftersom orsakerna till astma är svåra att diagnostisera, är det också svårt att veta vilken behandling som ska sättas in. En behandling som är effektiv för en patient kan vara ineffektiv för en annan.

Sjukdomar i små luftvägarna är svåra att studera eftersom de är svåråtkomliga för provtagning. Luftvägsträdets väggar täcks av ett tunt lager av vätska och idag saknas enkla och reproducerbara metoder för att samla in denna vätska. Vi tror att innehållet i denna vätska, kan användas för att studera inflammation vilket kan förbättra behandlingen av patienter.

Från vätskeskiktet som täcker luftvägarna bildas små luftburna droppar då vi andas. Dessa mycket små droppar kan sedan transporteras med utandningsluften. Det är därför möjligt att samla in prov med ursprung i små luftvägarna utan att behöva gå in i lungorna. Ett instrument med syfte att samla in och räkna dessa droppar har utvecklats vid Göteborgs universitet; instrumentet kallas för PExA.

I den här avhandlingen har detta instrument använts för att studera hur olika sätt att andas påverkar bildningen av dessa droppar, hur man bäst ska mäta innehållet i de insamlade dropparna och hur detta kan kopplas till inflammation.

Vi fann att metoden att samla in partiklar genom impaktion var mer effektiv än existerande metoder som bygger på att kyla utandningsluften. Vi fann att mängden partiklar som bildas varierar mycket mellan olika personer. Det är därför viktigt att justera provresultat för skillnader i insamlad provmängd, dvs mängd insamlade partiklar. Genom att andas med olika andningsmanövrar kan man styra vilken lungregion man får prov ifrån. Hos individer med mild astma och björkpollenallergi minskade mängden partiklar i utandningsluften under pollensäsong.

LIST OF PAPERS

This thesis is based on the following studies, referred to in the text by their Roman numerals.

I. Larsson P, Mirgorodskaya E, Samuelsson L, Bake B, Almstrand AC, Bredberg A, Olin AC. Surfactant protein A and albumin in particles in exhaled air. *Respiratory Medicine 106: 197-204, 2012.*

II. Larsson P, Lärstad M, Bake B, Hammar O, Bredberg A, Almstrand AC, Mirgorodskaya E, Olin AC. Exhaled particles as markers of small airway inflammation in subjects with asthma. *Clinical Physiology and Functional Imaging 2015. DOI: 10.1111/cpf.12323.*

III. Larsson P, Bake B, Wallin A, Hammar O, Almstrand AC, Lärstad M, Ljungström E, Mirgorodskaya E, Olin AC. The effect of exhalation flow on endogenous particle emission and phospholipid composition. *Submitted manuscript 2016.*

IV. Mirgorodskaya E, Larsson P, Koca H, Kim JL, Bake B, Ljungström E, Holm M, Olin AC, Exhaled surfactant protein A and albumin in a healthy Swedish population. *Manuscript.*

CONTENT

ABBREVIATIONS

Albumin	Human serum albumin
BAL	Bronchoalveolar Lavage
BSA	Bovine Serum Albumin
CI	Confidence Interval
COPD	Chronic Obstructive Pulmonary Disease
CV	Coefficient of Variation
DPPC	Dipalmitoylphosphatidylcholine
EBC	Exhaled Breath Condensate
ELISA	Enzyme-Linked Immunosorbent Assay
FENO	Fraction of Exhaled Nitric Oxide
FEV1	Forced Expiratory Volume in 1s
FRC	Functional Residual Capacity
GC-MS	Gas-Chromatography coupled to Mass Spectrometry
HEPA	High-Efficiency Particulate Arrestance
HRP	Horseradish Peroxidase
LoD	Limit of Detection
LoQ	Limit of Quantification
MS	Mass Spectrometry
MRM	Multiple Reaction Monitoring

OPC	Optical Particle Counter
OPD	O-phenylenediamine dihydrochloride
PBS	Phosphate-Buffered Saline
PEF	Peak Expiratory Flow
PEx	Particles in Exhaled air
POPC	Palmitoyl-Oleoyl-Phosphatidylcholine
PTP	Proteotypic Peptides
PTFE	Polytetrafluoroethylene
r_s	Spearman correlation
RI	Reference Intervals
RTLF	Respiratory Tract Lining Fluid
RV	Residual Volume
SD	Standard Deviation
SP-A	Surfactant Protein A
TLC	Total Lung Capacity
VC	Vital Capacity
VOC	Volatile Organic Compound
Vt	Tidal volume
wt%	Weight percent

DEFINITIONS IN SHORT

PEx	Particles in Exhaled air in the size range of 0.41–4.55 μm in diameter
Small Airways	Airways < 2 mm in internal diameter

INTRODUCTION

Lung diseases are among the leading causes of death in the world. Chronic obstructive pulmonary disease (COPD) is the third leading cause of death in the world according to WHO and asthma may affect as many as 300 million people in the world (1). This puts a great economic burden on society and results in loss of quality of life for those that are affected.

Asthma has traditionally been described as a result of chronic airway inflammation but the association between airway inflammation, airway dysfunction and degree of symptoms are poorly understood (2). This makes treatment of asthma difficult as individuals with similar symptoms respond differently to treatment. Because of the large heterogeneity in the underlying biology within the asthma group, asthma is often categorized into subtypes defined by different phenotypes. The difficulty in collecting biological samples from small airways (airways <2 mm in diameter) makes phenotyping of the small airways a challenge. A non-invasive method that can be used in the general population for sampling liquid from the small airways for quantitative analysis would open up new possibilities for phenotyping asthma, studying the inflammatory processes and guiding treatment.

Exhaled breath condensate (EBC) is likely the most used non-invasive method for collection a biological airway samples. Despite the large number of studies, the clinical usefulness of the EBC method has been limited. The main limitation of the EBC method has been the variable sample dilution and unknown sample origin (3).

Proteins and lipids are non-volatile and can only be transported in exhaled air as parts of a particle (i.e. droplet) in the aerosol. An instrument designed for sampling particles from exhaled air would likely result in more efficient and predictable sampling of the non-volatile components in breath than the EBC method.

In a collaboration between the Atmospheric Science section of the Chemistry and Molecular Biology department and the Department of Public Health and Community Medicine in Gothenburg, an instrument with the specific purpose of characterizing and sampling particles from exhaled breath was developed. The instrument samples exhaled particles by inertial impaction using a cascade impactor and was given the name PExA. By simultaneously measuring the number and size of sampled particles the sampled mass/volume of particles can be estimated. By measuring both the amount of analyte and the amount of

collected particles it is possible to determine the analyte concentration in the particles. The particle composition is likely to reflect the respiratory tract lining fluid (RTLF) composition from which the particle was formed. The first publication describing the PExA method was published in 2009 by Almstrand *et al.* (4).

The overall aim of the research presented in this thesis was to contribute to the development of measurements and analysis of exhaled particles towards a clinically valuable tool for early detection and monitoring of airway inflammation.

In the introduction of this thesis I will give a brief background description of airway physiology, particle formation, airway inflammation, and potential biomarkers. This background information is useful when designing the studies and interpreting the outcomes.

1.1 Airway physiology

We inhale around 10-25 m^3 of air to oxygenate our blood every 24 hours. The oxygen is required for the aerobic respiration that generates the energy needed to sustain life (5). The lungs have a structural design that is highly optimized for an efficient gas exchange between the inhaled air and blood. This requires a large interface area between blood and air, a short diffusing distance and an efficient and reversible binding of oxygen to haemoglobin in blood. There is approximately 200 mL of blood in the lung capillaries distributed over a gas exchange surface of around 130 m^2 (for a male of 70 kg) (6, 7) with a tissue barrier of around 1 μm (8, 9). This large surface area is created by only 300 g of tissue and fitted inside a volume of only 5 L (10).

1.1.1 Airway morphology

The airway physiology is extremely complex and simplified models are often useful when describing the airways. In 1963 Weibel *et al.* published a book "*Morphometry of the human lung"* that presents many of the concepts behind one of the most used lung models in airway physiology, referred to as Weibel's lung model (or model a) (6). The model divides the lung into three zones; the conductive, the transitional and the respiratory zone. The airways are described as a tree with 23 generations that branch by dichotomy (fig 1). The first sixteen generations constitute the conductive zone i.e. from trachea to terminal bronchioles. In the transitional zone, generation 16-19, the airway walls become increasingly lined with gas exchanging alveoli. The respiratory zone, generation 20-23, is made up of the alveolar ducts that terminate with the

alveolar sacs. The alveolar ducts are lined with gas exchanging alveoli and the alveolar sacs are clusters of around 10-15 alveolar units (7). At a lung volume of FRC the alveolar sacs have an estimated diameter of around 5 mm (7) where each alveolus has an approximate diameter of around 0.2 mm (11). The sizes of the alveolar sacs is likely to be quite similar between subjects at FRC, but the number of units increases with lung size (11). The total number of alveoli has been estimated to 300-800 million and the number of alveoli correlates to lung size (11, 12). For each generation of the conducting airways, the airway diameter is reduced by a factor of around 0.7–0.8 of the preceding generation. This is close to the ideal factor for avoiding dead space and minimizing work during respiration that has been calculated to 0.79 (13-15). Once the airway diameter reaches approximately 0.5 mm at the level of the terminal bronchioles, there is no further reduction in airway diameter in the following generations (16). The lung morphology has important implications for understanding particle formation and deposition during different ventilation cycles. Table 1 describes physical characteristics for each airway generation of Weibel's lung model and shows the velocity and residence time in each airway generation for a particle that transitions from the alveolus to the trachea, assuming an exhalation flow of 1.0 $L \cdot s^{-1}$. From table 1 it is clear that the lung volume is mainly localized to the distal airways. The last 7 mm of the airway contain approximately 96% of the total lung volume.

	Generation		Number of airways	Total crossectional area (cm^2)	Diameter (cm)	Length (cm)
Conductiong zone	trachea	0	1	2.54	1.80	12.0
	bronchi	1	2	2.33	1.22	4.8
		2	4	2.13	0.83	1.9
		3	8	2.00	0.56	0.8
	bronchioles	4	16	2.48	0.45	1.3
		5 ↓ 16	32 ↓ $6 \cdot 10^4$	3.11 ↓ 180.0	0.35 ↓ 0.06	1.07 ↓ 0.17
	terminal bronchioles					
Transitional and respiratory zones	respiratory bronchioles	17 18 19	↓ $5 \cdot 10^5$	↓ 10^3	↓ 0.05	↓ 0.10
	alveloar ducts	20 21 22	↓	↓	↓	↓
	alveloar sacs	23	$8 \cdot 10^6$	10^4	0.04	0.05

Figure 1. Weibel's lung model

	Airway	Generation	Nr of branches	Diameter (cm)	Length (cm)	Cross section (cm)	Volume (cm^3)	Cumulative volume (cm^3)	Velocity[1] ($m*s^{-1}$)	Residence time[1] (ms)
Conducting zone	Trachea	0	1	1.8	12.00	2.5	30.5	31	3.930	31
	Main bronchus	1	2	1.22	4.76	2.3	11.1	42	4.277	11
	Lobar bronchus	2	4	0.83	1.90	2.2	4.1	46	4.621	4
	Segmental bronchus	3	8	0.56	0.76	2.0	1.5	47	5.075	1
		4	16	0.45	1.27	2.5	3.2	51	3.930	3
		5	32	0.35	1.07	3.1	3.3	54	3.248	3
		6	64	0.28	0.90	3.9	3.5	57	2.538	4
	Bronchi with cartilage in wall	7	128	0.23	0.76	5.3	4.0	61	1.880	4
		8	256	0.186	0.64	7.0	4.5	66	1.438	4
		9	512	0.154	0.54	9.5	5.1	71	1.049	5
		10	1024	0.13	0.46	13.6	6.3	77	0.736	6
	Terminal bronchus	11	2048	0.109	0.39	19.1	7.5	85	0.523	7
	Bronchioles with muscles in wall	12	4096	0.095	0.33	29.0	9.6	94	0.344	10
		13	8192	0.082	0.27	43.3	11.7	106	0.231	12
		14	16384	0.074	0.23	70.5	16.2	122	0.142	16
	Terminal bronchiole	15	32768	0.066	0.20	112.1	22.4	145	0.089	22
		16	65536	0.06	0.17	185.3	30.6	175	0.054	31
Respiratory zone	Respiratory bronchiole	17	131072	0.054	0.14	300.2	42.3	217	0.033	42
		18	262144	0.05	0.12	514.7	60.2	278	0.019	60
		19	524288	0.047	0.10	909.6	90.1	368	0.011	90
		20	1048576	0.045	0.08	1667.7	138.4	506	0.006	138
	Alveolar duct	21	2097152	0.043	0.07	3045.5	213.2	719	0.003	213
		22	4194304	0.041	0.06	5537.5	326.7	1046	0.002	327
	Alveolar sac	23	8388608	0.041	0.05	11075.1	553.8	1600	0.001	554

Table 1. Weibel's model of airway dimensions for an average adult human lung with a volume of 4800 mL at about 3/4 maximal inflation (6). [1]:At a flow rate of 1.0 $L*s^{-1}$

1.1.2 The ventilation cycle

The airways are elastic structures that are connected to the chest wall via the pleura and the lung volume depends on the transpulmonary pressure, i.e. the pressure in the alveoli minus the intra-pleural pressure. At functional residual capacity (FRC) the elastic recoil properties of the lungs' tissue and surface tension are equal to the opposite and distending force of the chest wall. At FRC the transpulmonary pressure is about 5 cm H_2O and the intra-pleural pressure is negative (-5 cm H_2O). The pressure outside of the airways is approximately equal to the intra-pleural pressure (17). By expanding the chest wall and contracting the diaphragm the distending force becomes greater than the lung contractive force, the intra-plural pressure becomes more negative and the lungs will expand. This stretching force is opposed by the increase in recoil force of the lung's connective tissue (collagen and elastin fibres) and the surface tension force of the RTLF (18, 19). When the muscles are relaxed at high lung volume the contractive recoil force of the lung will provide the energy required for exhalation back to the FRC equilibrium. During tidal breathing inhalation is driven by energy input whereas the exhalation is passive return to FRC volume. The work required to inflate the lungs is related to the lung compliance. Compliance is the volume change per unit of pressure change. If the lungs have increased rigidity and resists a volume change, the compliance is decreased and a greater effort is required for ventilating the lungs. This can be seen in patients with pulmonary fibrosis where the rigid lungs may result in a reduced FRC volume (20). If the lung has increased compliance the recoil of the lung tissue is decreased and the distending force of chest wall will expand the lungs to a larger degree. This can be seen in patients with emphysematous destruction and an increase in FRC volume (20, 21).

1.1.3 Airway closure

The body position influences how the lung is ventilated. In a standing position the upper parts of the lungs will be stretched open to be more expanded than the lower parts due to the gravitational force (22). During a deep exhalation to residual volume in the upright position, small airways will close, particularly in the lower parts of the lungs where the lungs and airways are less expanded. The airways that close (and open) are thought to have a diameter of less than 1 mm, i.e. terminal bronchioles (23). Airway closure can be assessed by a simple single breath test, i.e. the closing volume test (24, 25). Closing volume denotes the lung volume at which massive airway closure begins.

1.1.4 Spirometry and lung volumes

Spirometry is a physiological test that measures how an individual inhales or exhales volumes of air as a function of time. The most common is the forced spirometry test that measures the flow over time during a maximal forced exhalation from total lung capacity (TLC) to residual volume (RV). The maximal expiratory flow is effort dependent at the very start (first 25% of lung vital capacity) but if the effort is sufficiently high the flow reaches a maximal value and an increased effort does no longer increase the exhalation flow. At this point the airway transmural pressure starts to become negative and the airways become compressed. In compressed airways the flow resistance increases and this creates a flow limiting choke point (fig 2). At this point the flow becomes decoupled from effort and is determined by the contractive force of the lung, determined by the lung tissue, the RTLF surface tension, and the flow limiting segment in the airways (the choke point). The maximal flow becomes effort independent but dependent on the choke point location. The choke point is generally found in central airways and spirometry is therefore more effective for measuring changes in central airways than changes occurring in small airways. Common lung volumes and their abbreviations are shown in fig 3.

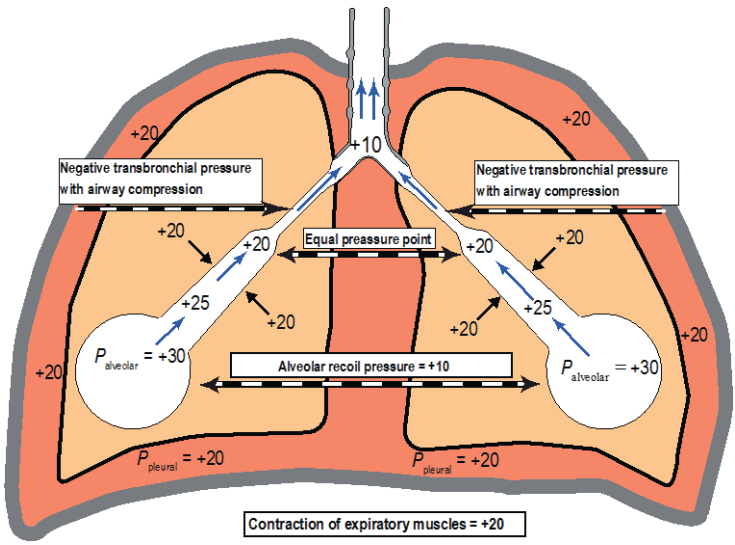

Figure 2. Dynamic airway compression, pressures are given as cm H_2O.

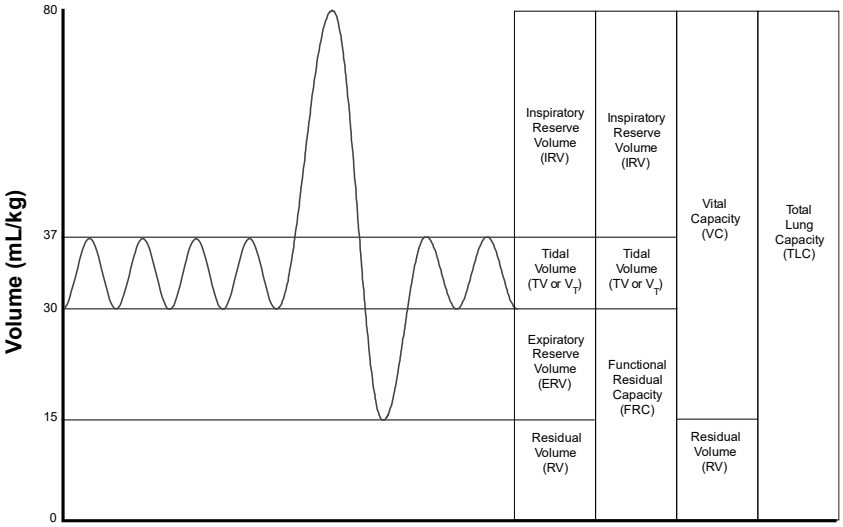

Figure 3. Ventilation cycle. From Wikimedia, uploaded by Vishadas and distributed under a CC-BY 2.0 license.

Respiratory volumes

- **Functional Residual Capacity (FRC)**: Volume remaining in the lung when the elastic recoil forces of the lungs are equal to the opposing chest wall recoil at relaxed breathing muscles.
- **Vital Capacity (VC)**: The maximum volume of air that a person can inhale/exhale. If the exhalation is fast it is called a Forced Vital Capacity (FVC) manoeuvre.
- **Total lung capacity (TLC)**: The volume of gas contained in the lung at maximal inspiration (TLC=VC+RV).
- **Tidal volume (V_t)**: Volume inhaled/exhaled during relaxed breathing.
- **Residual Volume (RV)**: The volume remaining after a maximal expiration.

RV can be measured using a body plethysmograph or a gas dilution test. If the RV is known, then the other volumes can be derived from a spirometry test.

Most commonly measured respiratory airflows

- **Forced Expiratory Volume in 1 s (FEV1):** Maximum volume exhaled from TLC in 1 s.
- **Peak Expiratory Flow (PEF):** Maximum flow rate of air at the mouth during a forced exhalation.

1.1.5 Epithelium and respiratory tract lining fluid

The distance from airway generation 0 to airway generation 23 is approximately 25 cm (7).

The main functions of the conductive airways are to keep the respiratory zone well ventilated with a minimum amount of effort and to protect the airways from harmful exposures. The main purpose of the respiratory zone is to facilitate the gas exchange of oxygen and carbon dioxide. To serve these different purposes the anatomy changes from the respiratory zone to the conducting airways.

There are many different types of epithelial cells in the airways (fig 4) (26). Mucin secreting goblet cells are present at almost all airway levels but decrease in the peripheral airways before disappearing in the terminal bronchioles. The ciliated epithelial cells are found all the way from respiratory bronchioles to glottis. The ciliated epithelial cells are responsible for the transportation of the RTLF towards glottis. The mucociliary transport is the main clearance mechanism of fluid and particles in the conductive airways under normal circumstances (27). In the trachea, the RTLF is transported towards glottis at a speed of 4–6 $mm \cdot min^{-1}$ by 6 μm long cilia that beat around 10 times per second in a coordinated action (28, 29). Because the airway cross section is decreasing towards glottis there would be a very large accumulation of fluid towards glottis if it was not for the absorption of liquid and the increased transportation velocity. In the major bronchi the RTLF speed is 2.6 $mm \cdot min^{-1}$ (29) and it is likely to further decreased towards the small airways. The non-ciliated club cells (formerly known as Clara cells) in the respiratory bronchioles are secretory cells that have protective functions. Club cells secrete large amounts of club cell protein 16 that is considered to protect against inflammation and oxidative stress (30). In the respiratory zone where there are no ciliated cells, the major clearance of particles is facilitated by the alveolar macrophages that phagocyte the material and transport it to either the ciliated part of the airways or to the lymphatic system. Phagocytosis by macrophages can be enhanced by opsonins e.g. surfactant proteins.

The alveolus is not the spherical structure that is often used in schematic illustrations but have a complex shape with flat sections and sharp curvatures between the walls (7). The walls are made up of the flat alveolar type I cells and cuboidal alveolar type II cells. The flat alveolar type I cell constitute around 95% of the alveolar surface area and are extremely thin (as low as 25 nm) to facilitate efficient gas exchange between air and blood. The type II cell is a major synthesizing and secreting factory whose main function is to produce

and recycle the pulmonary surfactant. The surfactant secreted by the type II cells is vital for the lung function and without surfactant the lung will collapse by the contractive force of the liquid lining the airways.

Studies on pulmonary surfactant are generally made on the surfactant retrieved from bronchoalveolar lavage (BAL). The BAL procedure dilutes the surfactant and the procedure itself may alter the surfactant composition. It can therefore be difficult to know how accurately the retrieved surfactant reflects the *in-vivo* composition in the alveolus. Surfactant is composed of around 90% lipids and 10% proteins by mass. The major lipid is the disaturated phospholipid dipalmitoylphosphatidylcholine (DPPC) that constitute around 40% of surfactant mass (31). DPPC has ability to reduce the surface tension between the air liquid interface to extremely low levels. The surfactant has the ability to alter surface tension dynamically with the changing size of the alveoli during respiration. As the alveolar size is reduced the surfactant is enriched in DPPC and the packing of DPPC becomes denser. The surface tension can be reduced to extremely low values of only 2–3 mN/m compared to the equilibrium surface tension of 22–23 mN/m (31, 32). The low surface tension at low lung volumes assists in reopening the airways and the increase in surface tension at high volumes assists in the passive exhalation back to FRC lung volume. In surfactant there is only around 7–10% of proteins by mass but they play an important role for surfactant function. There are four surfactant proteins SP-A, SP-B, SP-C and SP-D that constitute around 5.3, 0.7, 0.4 and 0.6% of pulmonary surfactant mass (31, 33), respectively. SP-B and SP-C are hydrophobic proteins that are essential for the spreading of surfactant at the fluid/air interface. SP-B is particularly important for surfactant function and SP-B deficient mice die at birth (34, 35). SP-A and SP-D are hydrophilic proteins belonging to the collectin family of proteins whose main function is thought to be in the innate immune system. In BAL fluid 95-99% of SP-A has been found to be bound to the DPPC in surfactant, whereas 75% of SP-D is found in the aqueous phase (7, 32, 33).

Figure 4. Airway cells and lining fluid. Reproduced with minor modification with permission from Anna Bredberg.

1.2 Airway Inflammation

Inflammation is the host response to a variety of insults; trauma, infections, toxins and disease to name a few. The purpose of inflammation is to remove the threat, remove dead tissue and initiate the tissue repair process. Even though there are many different types of inflammation they all share a number of common characteristics (36).

- Increased perfusion of blood
- Increased permeability of the vascular endothelia
- Leakage of protein to the extracellular matrix
- Recruitment of leukocytes from blood
- Severe inflammation lead to an adjusted metabolism and fever

One of the most typical characteristics of all inflammation is increased vascular permeability. Release of histamines and leukotrienes from the mast cells and formation of bradykinins cause endothelial cell contraction with widening of intercellular gaps in venules (37). Initially the leakage to the extracellular matrix is protein-poor (transudate) but if the gaps become larger a protein rich leakage (exudate) follows. This results in reduced intravascular osmotic/oncotic pressure and an increase in extravascular/interstitial osmotic pressure, resulting in an accumulation of fluid (edema) in the inflamed tissue.

1.2.1 Asthma

Asthma may affect as many as 300 million people in the world (1). The Global Strategy for Asthma Management and Prevention (GINA) defines asthma as ‘a heterogeneous disease, usually characterized by chronic airway inflammation”, and “by the history of respiratory symptoms such as wheeze, shortness of breath, chest tightness and cough that vary over time and in intensity, together with variable expiratory airflow limitation.’’ (38).

There are many so-called phenotypes of asthma, i.e. the observable differences in asthma characteristics, and each phenotype may have different underlying mechanistic pathways (39, 40), so called endotypes. At present there are no easy method to map the endotypes, nor any universally agreement upon classification of phenotypes.

The most common treatments to control asthma are inhaled short- and long acting beta2-agonist, that prevents airway constriction, and inhaled anti-inflammatory corticosteroids. These treatments are successful for managing asthma for many subjects, but less successful for others (41). One way to improve control may be to map the different endotypes (42). This approach has

been successful for the treatment of many types of cancer, however, it requires that the diagnosis can be made in an early stage before irreparable damage has been established.

1.3 Methods to study airway inflammation

1.3.1 Invasive methods

Biopsy

Biopsy is one of the few methods where the small airway tissue can be analysed, and is regarded as the gold standard.

The biopsy is an excellent sample for studying the inflammation process in the tissue but its use is limited by the invasive nature. Biopsies have the advantage of sampling a very specific airway region and give a specific knowledge about that region. If the inflammation is patchy a biopsy may miss the area with inflammation.

Blood

Serum concentrations of pneumo-proteins have been suggested as markers of airway inflammation (43-45). The hypothesis is that inflammation would increase the permeability of the alveolar-capillary barrier with a leakage of pneumo-proteins to blood, resulting in an increased serum concentration of these proteins. SP-A and CC16 are two proteins that are mainly produced in the small airways and have been suggested as potential markers of increased alveolar-capillary permeability in blood. Because of its small size and high mobility CC16 is often suggested as the preferred marker compared to the very large SP-A multimer that is associated to the pulmonary surfactant and less mobile. CC16 has been found to be changed after a number of airway exposures i.e. wood smoke (46), bacteria (47) and ozone (48). The serum concentrations of the pneumo-proteins are likely to depend on i) the leakiness of the alveolar-capillary barrier, ii) the concentration gradient (with diurnal variation) and iii) active clearance rate from blood (49). Because neither of these three processes is well understood it may be difficult to assess how the serum levels of a specific proteins is associated to inflammation.

Bronchoalveolar lavage

BAL is performed by instilling aliquots of saline into a selected bronchopulmonary segment with the use of a bronchoscope (50). The fluid that is recovered by aspiration contains both cells and acellular components from the peripheral airways. The BAL procedure is safe and less invasive than

biopsies but the use of bronchoscope requires anaesthetic and sometimes conscious sedation (51). Conventional BAL procedures aim to sample material predominantly from the respiratory region. The BAL method has many factors that can limit the reproducibility of the method (52). Sources of variability in the BAL method are:

- The recovery of the instilled solution varies, and may depend on disease, hence the dilution of retrieved RTLF may vary.
- It is difficult to know what region is being sampled.
- The BAL procedure itself can induce an inflammation and contaminate the sample with blood components.

Efforts have been made to standardize the BAL method (53) and the cellular component have proven to be a useful diagnostic tool for a number of airway diseases (50), particularly interstitial lung diseases (54). The acellular components of BAL are still of limited clinical value (55) due to the unknown sample dilution and unspecific sampling.

Induced sputum

The aim of sputum induction is to collect secretions from airways. Sputum production is induced by inhaling an aerosol of isotonic or hypertonic solution. The mechanism by which sputum is produced is not fully elucidated but it has been suggested that the increased osmolality increases the permeability of the bronchial mucosa and that sputum is secreted by the submucosal glands (56). Administration of radiolabelled aerosol boluses at different airway regions have shown that induced sputum mainly samples the central airways (57).

The limitation of induced sputum is that it requires administration of saline to induce sputum production (semi-invasive) and does not sample the peripheral airways. Similar to BAL, the acellular components of sputum are of limited clinical value (55) due to the unknown sample dilution and unspecific sampling. It is time consuming and can be unpleasant for the patient. In addition, sputum induction is not successful for all individuals (56). Despite these limitations, the use of sputum cell counts to guide asthma treatment have been found to reduce the risk of asthma acerbations in several studies (58-60).

1.3.2 Non-invasive methods

Exhaled breath is an aerosol that contains both non-volatile and volatile substances. The volatile substances behave as gas molecules whereas the non-volatiles are part of particles/droplets that are suspended in the gas. Because of the different physical behaviour of gas molecules and particles in an aerosol it is often useful to distinguish between volatiles and non-volatiles.

Non-invasive methods are very attractive because they can be used for screening the general population, be used for monitoring disease progression and to assess the effect of therapeutic intervention. The major limitations associated with the non-invasive methods are the low amount of material that can be collected, the unknown sample origin and the variable sample dilution.

Spirometry

Forced spirometry is the most common pulmonary test and is used in the clinical evaluation of most airway diseases. Spirometry is a lung function test that may detect the change in lung mechanics that arise as a result of inflammation. Spirometry is a sensitive method to detect central airway obstruction which is likely to affect the FEV1 measurement. FEV1 measurement is used for classifying the severity of COPD according to the GOLD criteria (http://goldcopd.org). Spirometry is also used to distinguish asthma from other obstructive lung diseases by performing a reversibility test. Where, a significant improvement in FEV1 after the intake of the bronchodilator is indicative of asthma. The major limitations with spirometry are that it cannot detect a disease before it has resulted in a lung function change and gives no information on what the cause is. In addition, spirometry has a low sensitivity towards measuring lung function changes in the small airways.

Fraction of exhaled nitric oxide (volatile)

Nitric oxide is produced throughout the airways and has several important functions in the airways: a stimulatory effect on submucosal gland secretion, regulating ciliary beat frequency (61) and is a potent dilator of blood vessels (62). During inflammation, higher than normal levels of nitric oxide are released from epithelial cells of the bronchial wall, where nitric oxide is synthesized from L-arginine by nitric oxide synthases (63). Increased fraction of exhaled nitric oxide (FENO) levels are strongly associated with asthma (64) and may predict the response to treatment with corticosteroids (65).

The measurement of FENO is flow sensitive and the exhalation flow has to be standardized. The recommended flow is 50 $mL \cdot s^{-1}$ (66) although higher flows are sometimes used as they are considered to provide measurements of the peripheral airway regions. Despite associations between FENO and inflammation, the clinical value of FENO measurements have not been sufficient for them to be recommended as a routine tool for guiding inhaled corticosteroid administration in individuals with asthma, according to the Cochrane review group (67). FENO is a non-invasive direct measurement but with limited clinical value apart from measuring type-II inflammation.

Volatile organic compounds

Exhaled breath contains thousands of volatile organic compounds that may reflect the metabolic processes in the airways (68, 69). The higher the vapour pressure of the solid/liquid the more volatile it is, with a higher tendency to transition into the gas phase. Molecules that have a comparatively high tendency to transition to the gas phase are referred to as volatile. There is no exact value for the vapour pressure required for a compound to be called a volatile organic compound (VOC), but a gradual transition. The short diffusion distance and the large area of the lungs means that the VOCs are efficiently released from the RTLF to air. There are many different types of VOCs and relatively little is known about their source. The golden standard to analyse VOCs is gas-chromatography coupled to mass spectrometry (GC-MS). Electronic noses are cheaper and easier to use alternatives to GC-MS. The electronic nose does not measure any specific compound but instead has sensors that interact with a wide range of compounds to create an array of electric signals that acts as fingerprints, or "breathprints" of the measured VOCs. Based on breathprints, electronic noses have successfully discriminated between subjects with asthma and controls in several studies, with reported accuracies of 89–100% (69-72). The classification is made by calibrating the instrument using a "training set" of VOCs from subjects with asthma and then cross-validate the instrument on a new set of individuals. To use the electronic nose for diagnosis, the instrument first has to be calibrated using a training set of individuals. It is not possible to produce two instruments with identical sensors having the same response and each instrument has to be individually calibrated and tested.

Exhaled breath condensate (volatile and non-volatiles)

Exhaled breath condensate (EBC) is a rapid, safe and non-invasive method to obtain a sample from the airways. The sample is obtained by cooling the exhaled breath and collecting the resulting condensate. EBC is likely to collect both volatile, semi volatile and non-volatiles. The recommended method of breathing is tidal breathing, which makes the sampling process easy to perform for the patient. There are a number of different types of EBC condenser. EcoScreen (Jaeger GmbH, Hoechbberg, Germany) is one of the most used stationary samplers with active cooling of the condensing chamber and the RT-tube (Respiratory Research, Inc., Charlottesville, VA, USA) is a smaller portable system that use a pre-cooled aluminium sleeve. The EcoScreen condenser tube has a temperature of around -20 °C and has a comparatively efficient condensation with recoveries of around 20–30% of the theoretical water volume (73) which is 0.045 mL per litre of breath (73, 74). Condensation is in theory a good method to collect semi volatiles that are in the gas phase in the lung and are cooled below the dew point in the condenser tube. Initially the

focus of EBC was the measurement of pH but a number of non-volatile substances such as proteins and lipids have also been found in EBC demonstrating that also particles/droplets are collected (3, 75-78). However, the EBC method is not designed for efficient particle sampling. With most EBC instruments, the sampling of non-volatiles is not efficient and the fraction of exhaled particles that are sampled is difficult to predict. It has been shown that both the condenser temperature and condenser coating influences the recovery of analytes (76). Anatomical origin of sampled EBC and mechanisms of EBC formation are largely unclear. Radioactive labelling of airway regions before sampling indicated that EBC mainly samples the central airways and does not consistently reflect the composition of small airway RLTF (79). The major limitation of EBC is the unknown sample origin, the variable and unknown dilution and the low concentration of analytes in the sample. Despite the large number of studies on biomarkers in EBC there are currently no clinical applications for the method.

1.4 Particles in exhaled air

In this section, non-invasive methods applied with the specific purpose of sampling particles for biomarker measurement are discussed in details. As was mentioned in the above section, the non-volatiles, e.g. proteins and lipids, are exhaled as particles/droplets that follow the air stream during exhalation. An efficient sampling method for non-volatiles in breath should focus on the collection of particles (74, 80, 81). An aerosol is a two-phase system of gas and condensed (solid/liquid) particles. A distinguishing difference between particles and gas molecules is that particles often adhere permanently to a macroscopic body if they make contact, a process referred to as deposition, whereas gas molecules bounce when they collide with a solid object. This means that particles can quickly be lost by deposition after they have formed and both particle formation and deposition have to be considered when studying particles in breath. In this paragraph, a short introduction to particle sampling, particle formation and particle deposition will be given.

1.4.1 Particle sampling methods

Electret filter sampling (SensAbues® device)

Several studies have demonstrated that particles can be sampled efficiently from breath using electrostatically charged filters. The electret filter material keeps permanent electrostatic charges and efficiently traps charged/polar aerosol particles from the air by electrostatic interaction (80, 82, 83). Electret filters can have a very low back pressure, compared with other filters, while

still having a high sampling efficiency. Because of the low back pressure, it is possible to exhale with very little effort through the electret filters, eliminating the need for an assisting pump. This makes it possible to construct very small sampling devices with a high sampling efficiency for particles in breath.

The SensAbues® collection device (SensAbues AB, Sollentuna, Sweden) is a small device developed around the electrostatic filter sampling technique with the main purpose of drug testing (82). The collected particles contain DPPC which indicate presence of pulmonary surfactant (83, 84). This makes the device interesting as a research tool for collecting fluid from small airways. The SensAbues® device samples particles close to the mouth and likely collects particles over a large size range. This has the advantage that more material can be sampled but it cannot be assumed that the sample originates exclusively from the small airways. Exhaled particles from the small airways are unlikely to be larger than 5 μm (85). A limitation with the SensAbues® collection device is that the amount of sampled particles is not measured. It is therefore not possible to relate measured concentrations to sampled amount of particles.

Inertial impaction sampling (PExA method)

The PExA instrument samples exhaled particles by impaction and characterises the particle number concentrations and size of particles using an optical particle counter (OPC).

Impactors use the principle of particle inertia to sample particles according to size (86). The aerosol is drawn through nozzles using a vacuum pump. The jet of air that exits the nozzle is directed towards an impaction plate. The impaction plate deflects the flow to give a 90° bend in the streamlines. Particles with inertia above a certain threshold will impact whereas particles below the threshold will follow the streamlines around the impaction plate. For particles of the same density, the particle inertia is mainly determined by particle mass, i.e. size and particle velocity. The size range of particles that impact on the impaction plate can be controlled by adjusting the velocity of the air stream.

Because the size ranges of particles that is sampled on the impaction plate is well-defined, the sampled mass of particles on the impaction plate can be calculated from particle measurements made with an OPC. The mass of sampled biomarkers on the impaction plate can be measured using chemical methods. When both the sampled mass of biomarker and the sampled mass of particles are known it is possible to calculate the concentration of biomarker in the particles. The concentration of biomarker in the particle is constant whereas the concentration of biomarker on the impaction plate is to a large extent determined by the amount of sampled material. Measuring the sampled particle

mass with an OPC may therefore solve the problem of unknown and variable sample dilution.

The major limitation with the PExA method is that the amount of sample that can be collected from breath is small compared to the invasive methods. Compared to the SensAbues® collection device, the cost of the PExA instrument is much higher, the instrument is less portable and it requires skilful operation.

1.4.2 Particle formation during breathing

Forced exhalation

During forced exhalations shear forces between RTLF and the air are created as a result of airflow, airway wall vibrations and dynamic airway compression (87). This interaction between the fluid on the airway wall and the air creates a two-phase concurrent flow (88, 89). If the air velocity exceeds 25 $m \cdot s^{-1}$ then a so called mist flow of particles and gas can be produced (88). During a forced exhalation, the highest air velocity is expected at the choke point created by dynamic airway compression (90). This choke point is generally formed in the region around the segmental bronchi (airway generation 3), where the total cross-section of the airways is the smallest (approximately 2.0 cm^2 for a lung of 4.8 L at 75% lung capacity) (6), but may also progress further peripherally at very low lung volumes (90). Exhalation flows during forced exhalation can exceed 10 $L \cdot s^{-1}$ and the total cross section of the central airways may be reduced to one sixth of its normal value by dynamic airway compression (91). The resulting air velocities can be extremely high and speeds over 120 $m \cdot s^{-1}$ have been measured in human trachea (92), even higher velocity is to be expected in the more compressed segmental bronchi.

However, air velocity is not the only determinant of particle formation during forced exhalations. Determinants for mucus clearance, according to a study by King *et al.* using a "cough simulating instrument," are air velocities, mucus viscosity, and mucus depth (93). In normal airways where the mucus depth is very low (below 80 μm) (94, 95) particle formation is expected to be low even when high air velocities are generated (89). Cough and forced exhalations appear to be effective for mucus clearance only when the mucus thickness reaches a certain level, as can occur with extensive mucus production and/or impaired ciliary transport (27, 96). Despite the high air velocities in the central airways the air velocities in small airways remain low due to the much higher total cross-sectional area. There is also less turbulence since the individual airways have a much smaller diameter. The shear forces on the fluid in the small airways are therefore considered to be very low and particle formation

by this mechanism to be unlikely. Even though forced exhalations have been suggested to be an efficient way of increasing the exhaled amount of particles and increasing mucus clearance in central airways there is very little experimental support for this. A study by Johnson *et al.* found that the increase in particle emission by using a faster exhalation flow was small (97). Particle formation associated with high shear forces during exhalation may thus be an overestimated mechanism for particle formation in healthy subjects.

Cough

During cough the emission of particles is clearly visible. Cough has three defining features: an initial deep inhalation, a brief powerful expiratory effort against a closed glottis and an opening of the glottis with a vigorous exhalation through the mouth (88). Cough is a crucial defence mechanism for expelling foreign bodies from the airways. Cough can be initiated both voluntarily and by a reflex. The exhalation flow during cough may exceed the flow attained during forced exhalations due to the built up pleural pressure preceding the cough, but the flow quickly becomes limited by dynamic airway compression. The high particle emission by cough is well documented but the airway region where particles are formed and the mechanisms for this are not well known. Usually the same particle formation mechanisms are suggested for cough as those suggested for forced exhalation but cough may also include additional particle forming mechanisms that are not yet described.

Airway closure and reopening

A study by Almstrand *et al.* demonstrated that particle emission could be increased dramatically without using high exhalation flows that cause high shear forces (98). When exhalations are preceded by an inhalation from a very low lung volume, the emitted amount of particles increase dramatically in the following exhalation. Since the exhalation flow is very low, particle formation by shear forces between liquid and air could be excluded. The suggested mechanism for particle formation is film bursting of RTLF that occurs when closed airways reopen during inhalation. During the reopening of small airways, a meniscus of RTLF is formed between the airway walls. When this meniscus ruptures, the surface tension force accelerates the liquid at the rim of the bursting film by a significant force, strong enough to generate particles (fig 5) (99-101). This mechanism of particle formation during inhalation is supported by the observed decline in exhaled amount of particles when the exhalation is delayed (102). When the exhalation is delayed particles settle by gravitational deposition and fewer particles are exhaled, indicating that the particles are formed during the inhalation. The particle formation as a result of airway opening has been supported by the results in several studies (85, 97, 102, 103). The chemical composition of particles emitted by this breathing

manoeuvre is characteristic of small airways, where airway closure is most likely to occur (104). Despite the large inter-individual variability in exhaled particle number concentrations following an airway closure and reopening manoeuvre, the size distribution is conserved. The size distribution of exhaled particle following an airway closure and reopening manoeuvre is shown in fig 6.

Tidal breathing

Several studies have demonstrated that particles are also emitted during tidal breathing, but for most individuals the emission was very low (97, 105, 106). The source of these particles is not fully known but there is increasing evidence that these particles are also formed by airway opening in small airways. Several studies have demonstrated that the emission of particles increased with increasing tidal volume (85, 106, 107). Ventilation volume is likely to also increase the number of closing and reopening airways indicating that the particles are formed by this mechanism. This suggests that there is a small degree of airway closure and reopening during tidal breathing. The size distribution of exhaled particle during tidal breathing is shown in fig 6.

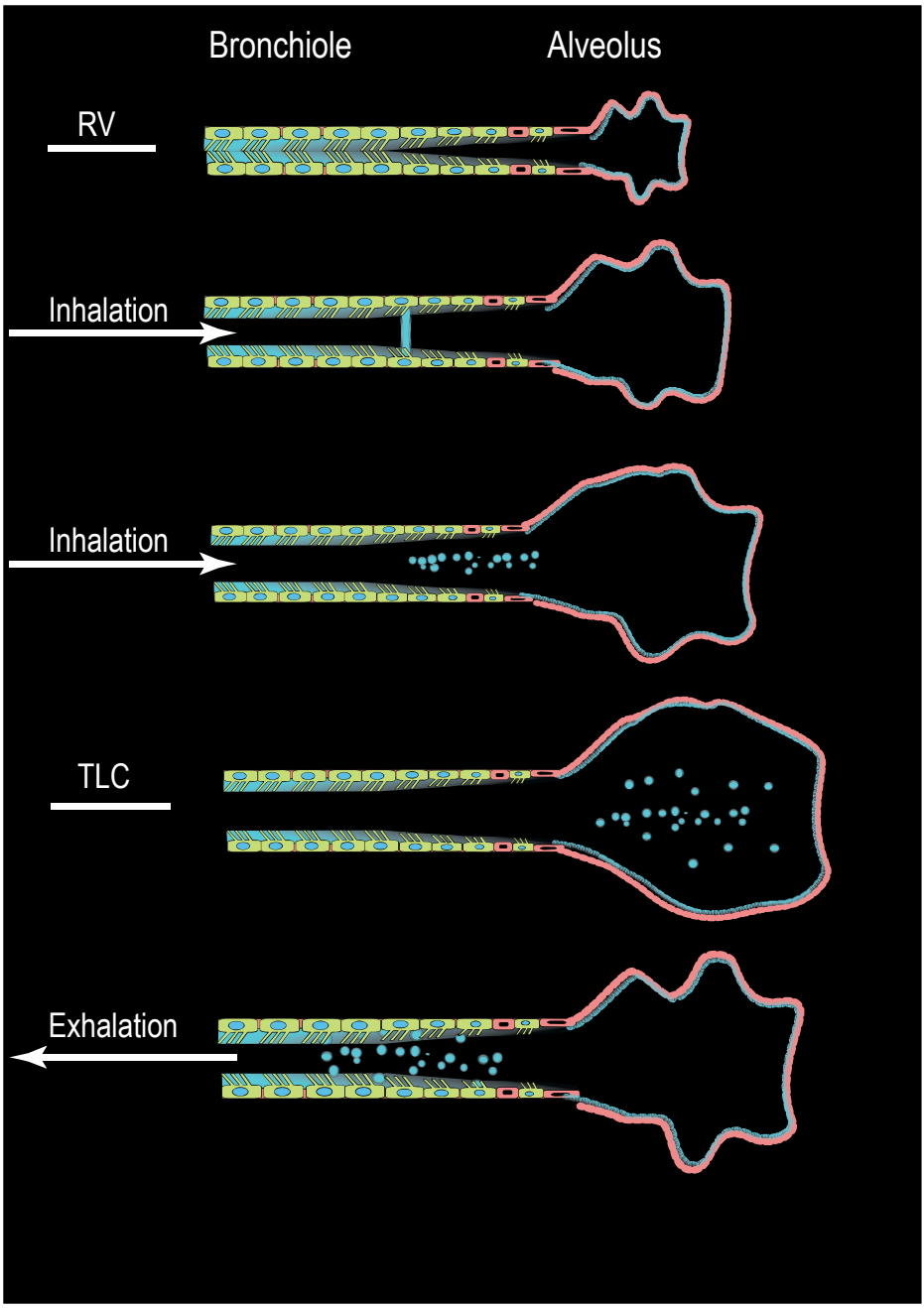

Figure 5. Airway closure and reopening

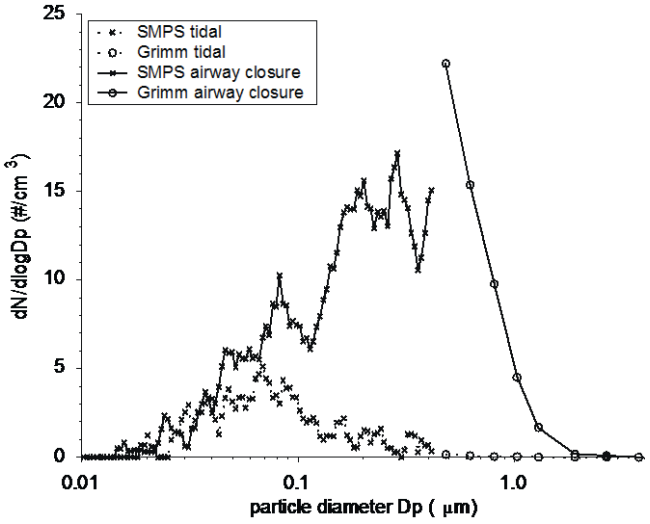

Figure 6. Average size distribution of PEx measured in 16 individuals, adapted from Holmgren et al. (101). SMPS: scanning particle mobility sizer; OPC: optical particle counter

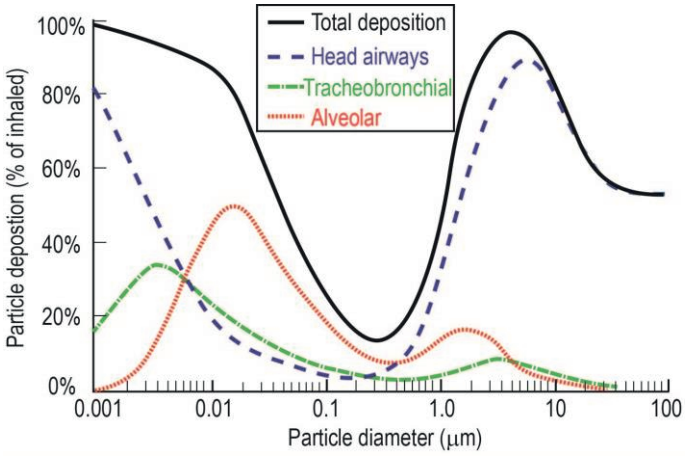

Figure 7. Airway deposition of inhaled particle based on the ICRP deposition model for light exercise nose breathing (333 mL·s^{-1}), average data for males and females (108).

1.4.3 Particle deposition

The exhaled particle number concentrations and size distribution of particles are influenced by particle deposition in the airways (86).

The five main particle deposition mechanisms are:

- Gravitational settling/sedimentation: A result of the gravitational force that acts on the particles. The particles fall in the gravitational field until they contact a solid surface and deposit. This mechanism is particularly efficient for large particles with a high density as these particles have a higher terminal falling velocity. This is a particularly important mechanism in small airways.
- Impaction: When particles deviate from the gas stream line due to high moment of inertia and impact on the surface of a solid object rather than follow the gas stream line around the object. This mechanism is particularly efficient for particles that have a high mass and a high velocity. It is particularly important mechanism in central/upper airways.
- Diffusion/Brownian motion: Occurs as the particle moves around due to collisions with the gas molecules. It is particularly important mechanism for small particles < 0.1 μm.
- Interception: Occurs when the particle stream line intercepts a solid surface and the particles adheres to the surface. This can be an efficient deposition mechanism for particles that have a large surface area.
- Electrostatic attraction: The attraction between a charged surface and an oppositely charged particle. This is not generally an important mechanism in the airways but can be used to collect exhaled particles.

The main deposition mechanisms acting in the airways are diffusion, sedimentation and impaction. Which of the three deposition mechanisms that is the most important is influenced by the properties of the aerosol, the airway geometry and the ventilation parameters. In addition to deposition, particles can be lost by coagulation i.e. when small particles collide to form larger particles. The complexity of the interaction between airway morphology, ventilation cycle and aerosol properties makes it difficult to model particle deposition even though the physical mechanisms are well described. In the upper and central airways, the air velocity can be high as a result of the small

total cross-sectional area of the airway and the main deposition mechanism in this region is impaction. In the small and peripheral airways, the main deposition mechanism is gravitational settling (and diffusion for particles 0.1 μm) due to the short distance between particles and the airway wall. During ventilation with low flow rates particle deposition is increased mainly in the small airways by gravitational settling, whereas during forced exhalation the deposition by impaction is increased in central and upper airways. The deposition region of particles in the airways varies with different ventilation parameters, however, in general for particles of 0.1–1.0 μm the deposition by both impaction or gravitational settling is relatively low. The size distribution of exhaled particles following an airway closure reopening manoeuvre (fig 6) is almost an inverse of the deposition profile (fig 7). This suggests that the observed size distribution of exhaled particles is likely a result of deposition in the airways rather than reflecting the size distribution of particles at the formation site. To be able to measure the chemical composition of particles, it is important to sample as high mass of particles as possible. The mass distribution has a mass median aerodynamic diameter of around 1 μm and since both the particle mass and the number of particle decline rapidly below 0.4 μm the total mass for particles below this diameter is very low. Particles larger than 2.0 μm are few but can carry a significant mass, a particle with a diameter of 5.0 μm carry around 1000 times the mass as a particle of 0.5 μm. However, particles larger than 7.0 μm are unlikely to originate from small airways (85).

1.4.4 Potential biomarkers

WHO defines biomarker as "almost any measurement reflecting an interaction between a biological system and a potential hazard, which may be chemical, physical, or biological. The measured response may be functional and physiological, biochemical at the cellular level, or a molecular interaction." (109). The selection of potential biomarker in exhaled breath is often limited by the small amount of collected material. Potential biomarkers for the PExA method will be further described here.

Exhaled particles

Both particle number concentrations and the size distribution of exhaled particles are potential biomarkers. Particle formation could be altered by lung properties that are influenced by biological processes. Examples of lung properties that are relevant for particle formation are the mechanical properties of the airways, the morphology of the airways and fluid properties of the RTLF. The surface tension of RTLF and the viscosity have been shown to influence the particle formation processes (85, 110). There is a very large inter-

subject variability in the exhaled number of particles in healthy subjects and it would be difficult to establish a normal range and a pathological range of values that could be used for diagnostic purposes. The intra-subject variability is however much lower if specific breathing manoeuvres are used. Particle number concentrations may be useful for monitoring changes in the airways over time or before and after an airway provocation e.g, a bronchial challenge test.

SP-A

SP-A has many properties that make it interesting as a potential biomarker for small airway disease. It is the most abundant of the surfactant proteins and one of the more abundant proteins in particles sampled from exhaled breath (104). This means that the amount of particles required for SP-A analysis is relatively low and breath sampling period can be reasonably short. In the airways, SP-A is mainly expressed in the type II cells and the non-ciliated bronchiolar club cells from two different genes SFTPA1 and SFTPA2, with the gene products SP-A1 and SP-A2 (33). Three SP-A chains are linked by cysteine bonds to form a trimeric unit. These trimeric units can form a hexamer structure with a molecular weight of 630 kDa (18 chains). The SP-A1 and the SP-A2 have four regions: the carbohydrate recognition domain, the α-coiled neck domain, the collagen like domain and the cysteine-rich N-terminal (fig 8) (33, 111). Mature SP-A consists of a hexamer of trimers that binds cooperatively via its globular carbohydrate recognition domain (CRD) to sugars and phospholipids. The binding capacity of SP-A is Ca^{2+} depended and in the absence of Ca^{2+} the binding affinity for the ligand is reduced (112, 113). In humans, almost all SP-A is found in alveoli and very little in the respiratory epithelium of the conductive airways (114). SP-A in blood is considered to be a result of alveolar leakage and the concentration in blood is much lower than in the alveoli (7, 33, 115). SP-A and surfactant have been observed to organize into structures called tubular myelin (32). This suggests that SP-A may have functions for the surfactant homeostasis although the physiological benefit of this has not been shown. Knock out mice lacking SP-A have not been found to have any surfactant dysfunctions (116, 117). SP-A is secreted by the type II pneumocytes and club cells. Therefore, SP-A concentration in RTLF could potentially be a biomarker for dysfunctional type II pneumocytes or club cells. SP-A have been proposed to have many different functions and some have been validated while others are more uncertain (33). The host defence roll appears to be the major function. Studies on SP-A knock out mice showed that they are more susceptible to infection to both viruses and bacteria (33). SP-A agglutinates a wide variety of pathogens which facilitates their uptake by macrophages. SP-A also modulates the adaptive immune response through its interaction with leucocytes (117, 118). SP-A has been found to modulate the

allergic inflammation in asthma by affecting the binding between antigen and IgE. An interesting hypothesis is the "head or tail hypothesis" where binding between the SP-A head to immune cells is anti-inflammatory whereas binding of SP-A tail to immune cells is pro inflammatory (111). There is a growing understanding of the many different roles of SP-A for maintaining immunological homeostasis in the lung. SP-A is involved in a wider range of biological processes that are associated with inflammation in peripheral airways, which makes it an interesting candidate for biomarker research.

Albumin

Albumin is one of the major proteins found in exhaled particles with a concentration exceeding that of SP-A (119). Human serum albumin (albumin) is transcribed from a single copy gene divided into six domains on chromosome 4 (fig 8). Albumin has a mass of 65 kDa and is mainly produced in the liver before it is secreted into the blood. Albumin constitutes around 50% of the plasma proteins in normal healthy individuals with a normal concentration range of 35–50 $g \cdot L^{-1}$, i.e. 3.5–5.0 weight percent (wt%). Some functions of albumin are to transport substances in blood, act as an anti-oxidant and to regulate fluid balance in tissues (120). The flow of water between compartments in the body is determined by both the osmotic force and the hydrostatic force. The osmotic force, determined by both ions and colloids, can be regulated to oppose the hydrostatic force to control flow of water even in the presence of a hydrostatic pressure difference (7, 121). Albumin is the main determinant of plasma oncotic pressure (80%) and plays a pivotal role in modulating the distribution of fluids between body compartments (120). Albumin circulates from the blood across the capillary wall into the interstitial compartments and returns to the blood through the lymphatic system with a circulation half-life of approximately 16 hours, the exchange rate is around 5% per hour (120). Albumin interstitial concentration is approximately half that of plasma but the concentration in RTLF is not well known (120).

Almost all airway diseases are associated with inflammation. One of the main characteristics of all inflammation is plasma exudate to surrounding tissues. The plasma exudate from blood is likely to disrupt the fluid balance in the airways and plasma proteins leaking into the airways could disrupt the surfactant function (44, 45, 122, 123). The association between plasma protein leakage and increasing inflammation in subjects with asthma have been demonstrated in several studies (44, 45, 122). Often BAL is used to measure proteins in RTLF but the results can be unreliable since the installation of saline into the airways could alter the proteins composition and the dilution of the recovered RTLF is unknown. The invasive nature of the BAL also limits the potential of BAL to study leakage of plasma proteins in the general

population as it cannot be used routinely or repeatedly. The effect of inflammation and increased vascular permeability on albumin concentration in RTLF is not as straight forward as it first appears. The permeability can be size selective with high permeability of small molecules and a lower permeability of large molecules (124, 125). In high-permeability edema the alveolar plasma protein concentration can approximate the plasma concentration whereas for hydrostatic edema the ratio is generally less than 0.6 (7). Increased hydrostatic pressure without an increase in albumin permeability could potentially lower the albumin concentration in the peripheral airways. It is also possible that albumin is actively transported out from the airways to oppose an increase in hydrostatic pressure difference. Therefore, both the hypothesis of albumin increase and the hypothesis of albumin decrease in exhaled particles as a result of inflammation should be considered.

DPPC and POPC

Phospholipids are the main component in particles originating from small airways and measurable levels are reached in a few breaths. In the airways, DPPC is only known to be produced in significant amounts by type II pneumocytes (126-128), constituting around 40–60 mol% of the phospholipids in surfactant that is purified from BAL samples (126, 129-134). The majority of the remaining lipids are unsaturated (32). The lipid composition in the surface layer changes during the ventilation cycle. The lowest surface tension is observed at low lung volumes where the DPPC component in the surface layer is highest. Surfactant have been found also in the central airways (28, 135). This surfactant is likely produced in the alveolar region and transported to the central airways by the mucociliary transport rather than secreted from the underlying mucosa (126, 128). The DPPC concentration is expected to be highest in the alveoli where it is produced and to decline further up in the airways due to dilution, absorption and breakdown. The amount of surfactant that reaches the central airways is not well documented but is likely to be low (7). Pettenazzo *et al.* estimated that around 7% of instilled and labelled phosphatidylcholine reached the glottis by the mucociliary clearance mechanism (136).

Palmitoyl-oleoyl-phosphatidylcholine (POPC) is the most abundant of the unsaturated lipids and constitute around 10 mol% of the phospholipids found in surfactant purified from BAL (126). POPC is not specific for surfactant and is a common component of most cell membranes. In surfactant DPPC molar concentration is around five times that of POPC whereas in airway mucosa and saliva POPC concentration is higher than the DPPC concentration (83, 126, 128).

Surfactant has a very specific function in the alveoli, to modulate surface tension and prevent airway collapse at the end of expiration (137). This function likely requires that the surfactant has a certain composition that is conserved between individuals. This could explain why there is a relatively low variability in surfactant lipid composition between individuals (126, 133, 134) and most animals have a similar composition (131, 132). This indicates that altered DPPC composition is likely to be associated to airway dysfunction.

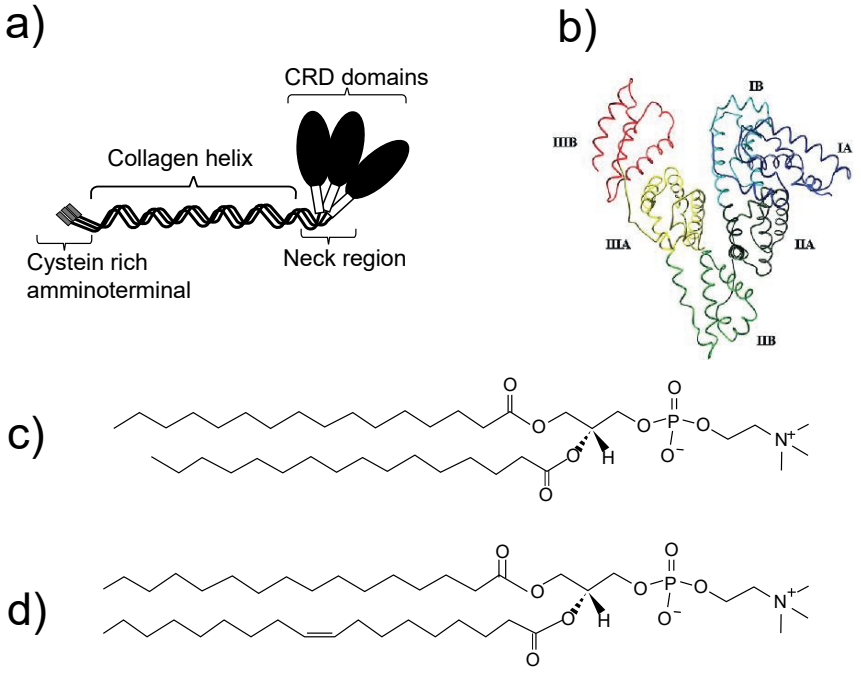

Figure 8. The four potential biomarkers that are studied in this thesis. a) SP-A with domain structure. The CRD-domain that binds to pathogens and allergens, the flexible neck region, the collagen helix region and the cysteine rich region where the subunits are linked. b) Human serum albumin structure. c) DPPC lipid structure. DPPC has two 16 carbon chains without double bonds. d) POPC lipid structure. POPC has one 16 carbon chain and one 18 carbon chain having one double bond.

2 AIMS

The overall aim of this thesis was to contribute to the development of a non-invasive method for sampling of RTLF with the purpose of studying airway inflammation.

- *Paper I (proof of concept):* To test applicability of the PExA method for monitoring the protein composition in RTLF from small airways.

  The specific research questions were:

  - To determine if SP-A and albumin can be analysed in PEx samples.
  - To compare the PExA method to the EBC method.
  - To determine if there is an association between sampled SP-A and albumin amount to sampled PEx amount.
  - To investigate the correlation between SP-A in PEx and SP-A in blood.

- *Paper II (clinical study):* To test the hypothesis that the PExA method could be used to study the effect of birch pollen-induced inflammation in individuals with mild asthma and birch pollen allergy.

  The specific research questions were:

  - Is birch pollen exposure associated with SP-A and albumin concentration in PEx?
  - Is birch pollen exposure associated with exhaled PEx mass concentration?
  - Is exhaled PEx mass concentration associated with FENO?

- *Paper III method development:* To study how different breathing manoeuvres influence particle emission and composition.

- *Paper IV normal values for healthy individuals:* The aims were to estimate reference intervals (RI) for SP-A and albumin in PEx for a population without lung disease and to investigate relationships between the observed protein concentrations and atopy, age, gender, anthropometry as well as spirometry data.

3 MATERIALS AND METHODS

3.1 Method development and validation

Prior to this PhD study very little experience on PEx sample preparation for enzyme-linked immunosorbent assay (ELISA) analysis was available. Substantial method development steps had to be carried out to achieve the aims of this thesis. Method development and validation was done continuously to address questions for the planned studies. Knowledge obtained in the completed studies was adopted in the following studies. The method modifications and validation steps made between the papers are presented in chronological order in appendix A.

3.1 PEx definition

The PExA instrument measures and samples particles in the size range of 0.41–4.55 μm in diameter. These particles are defined as PEx and the collected particles are referred to as a PEx sample.

3.2 Population and study design

3.2.1 Paper I

Pilot study

The protocol developed for paper I was first tested in a small pilot study of five individuals. PEx and EBC were sampled from 100 L of exhaled breath. PEx samples were sampled using an airway closure and reopening breathing manoeuvre. EBC was collected using tidal breathing. In PEx samples, SP-A and albumin were analysed and in EBC samples only SP-A was analysed.

Paper I

Population

Five men and five females volunteered to participate in the study. All participants had normal lung function based on a questionnaire and spirometry. One female was excluded from the study due to difficulties to perform the breathing manoeuvre, resulting in a very low emission of particles. This individual also had difficulties to perform deep exhalations during the spirometry test. The study protocol was approved by the Ethical Review Board of the Medical Faculty at the University of Gothenburg, Sweden.

Design of experiment

PEx, EBC and serum samples were collected during a single session between 09:00 and 12:00 to minimize effects of circadian rhythm. To study the variability between two sampling sessions a second sampling session was performed within a week of the first. PEx was collected from 100 L of exhaled breath using a three phase breathing manoeuvre where only the final exhalation was sampled.

1. Deep exhalation to residual volume
2. Full and rapid inhalation to total lung capacity
3. Exhalation from total lung capacity to residual volume with a flow rate of 1000–1500 $mL \cdot s^{-1}$

EBC was collected from 100 L of breath using tidal breathing according to manufacturer instructions. In addition, three EBC samples were collected using the same three phase breathing manoeuvre as used for PEx sampling. Sampled SP-A and albumin masses were determined by ELISA.

Outcomes

- Total amount of SP-A and albumin collected from 100 L of breath
- SP-A and albumin variability between sampling sessions and between subjects
- Correlation between exhaled SP-A and serum SP-A
- Correlation of sampled PEx mass to sampled SP-A amount and albumin mass

3.2.2 Paper II

Population

Five females and eight males diagnosed with mild intermittent asthma were recruited to participate in the study. They were all non-smokers with self-reported allergy symptoms. Birch pollen allergy was confirmed by measuring IgE antibodies against birch pollen in blood and seven individuals were also positive for at least one other antigen in the Phadiatop test (grass/mite/dog/cat/horse) (Pharmacia & Upjohn Diagnostic, Uppsala, Sweden). They reported no or mild symptoms outside of pollen season and were clinically stable. Six subjects were using inhaled glucocorticoids and four subjects were using long acting β2 stimulators. One subject was using both corticosteroids injections and oral corticosteroids. The participants were asked not to use long acting β2 stimulators for two days before the examination. A

control group with five females and eight males were recruited for the study. Individuals in the control group were non-smokers, with no history of lung disease and were negative for all antigens in the Phadiatiop test. Baseline characteristics and changes between the seasons were compared between the two groups. The study protocol was approved by the Ethical Review Board of the Medical Faculty at the University of Gothenburg, Sweden and all participants gave their written informed consent.

Design of experiment

All subjects were examined once outside of pollen season and once during pollen season. Each examination included:

- Spirometry before and after bronchodilation
- $FENO_{0.05}$, $FENO_{0.10}$ and $FENO_{0.27}$
- PEx sampling before and after bronchodilation

Outcomes

- PEx mass per litre of breath
- SP-A and albumin concentration in PEx
- Correlation of sampled PEx mass to sampled SP-A and albumin mass
- FENO at different exhalation flows: $FENO_{0.05}$, $FENO_{0.1}$ and $FENO_{0.27}$

3.2.3 Paper III

Pilot study

Population

Ten volunteers, four men and six women, from the department of occupational and environmental medicine were included in the pilot study. All participants were non-smokers with normal lung function based on a spirometry tests. The study protocol was approved by the ethics committee of Sahlgrenska Academy, University of Gothenburg.

Design of experiment

Two breathing manoeuvres that were expected to generate particles by different mechanisms in different airway regions were designed. In addition, a reference manoeuvre with slow exhalations and a low degree of airway

closure/reopening was included as a baseline measurement of particle emission. The breathing manoeuvres are shown in fig 9.

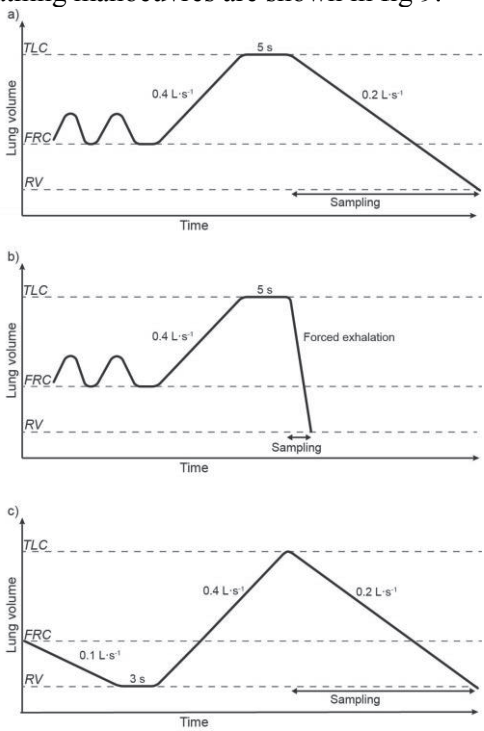

Figure 9. Breathing manoeuvres used in pilot study. a) The reference manoeuvre used a slow exhalation, designed to generate few particles and to estimate baseline for PEx emission and SP-A content. b) The forced exhalation manoeuvre with a maximally high exhalation flow, designed to induced particle formation in proximal airways during exhalation by the flow-mucus interaction. c) The airway opening manoeuvre, designed to generate particles in small airways during inhalation by the airway opening mechanism, these particles were expected to have the highest concentration of SP-A.

Outcome variables

- Exhaled PEx and SP-A mass per litre of exhaled breath
- SP-A concentration in PEx

Paper III

Based on the observations during the pilot study, the study design and the instrumental setup were slightly changed between the two studies.

Population

Eleven volunteers, 6 men and 5 females, with normal lung function based on spirometry participated in the study. There were ten non-smokers and one smoker in the study group. All participants gave their written informed consent, and the study protocol was approved by the Ethical Review Board of the Medical Faculty at the University of Gothenburg, Sweden.

Design of experiment

Particles were sampled by the PExA method using the four different breathing manoeuvres depicted in fig 10.

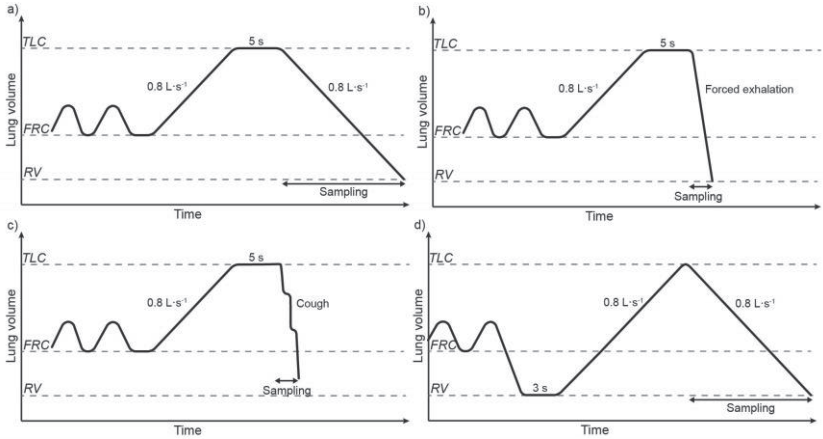

Figure 10. a) The FRC-reference manoeuvre used a slow exhalation and was designed to generate few particles and to estimate baseline for PEx, DPPC and POPC mass concentrations in exhaled air. b) The forced exhalation manoeuvre used a maximally high exhalation flow and was designed to induced particle formation during exhalation by the flow-mucus interaction. c) The cough manoeuvre was included as it is expected to produce very high particle emissions by various mechanisms. d) The RV-reference manoeuvre was designed to generate particles in small airways by airway closure and reopening, these particles were expected to have the highest concentration of DPPC.

Emission of PEx, DPPC and POPC mass per litre of breath was evaluated by comparing to the FRC-reference manoeuvre. DPPC and POPC concentration in PEx was compared to the RV-reference manoeuvre. The sampled mass of DPPC and POPC was determined with a triple quadrupole mass spectrometer equipped with an electrospray ionization ion source using a selected reaction monitoring method.

Outcome variables

- PEx, DPPC and POPC mass per litre exhaled breath
- Concentration of DPPC and POPC in PEx

- Correlation between sampled PEx mass to DPPC and POPC mass
- Ratio of DPPC to POPC
- Size distribution of exhaled particles

3.2.4 Paper IV

Population

Healthy individuals were selected from the 342 participants of the European Community Respiratory Health Survey (ECRHS) III, examined at the Gothenburg center during 2011-2012. The ECRHS III participants were randomly selected from the population in the Gothenburg area, but were enriched by subjects reporting asthma symptoms. The study was approved by the Central Ethical Review Board in Uppsala, as a part of a national study.

Healthy individuals were selected based on the ECRHS III questionnaire, after exclusion of current smokers. All subjects that had current asthma or answered positive to doctor diagnosis of either 1) chronic bronchitis, 2) COPD, 3) emphysema, or 4) any other lung disease were excluded. Current asthma was defined as doctor-diagnosed asthma, plus currently taking asthma medication or asthma attack in the last 12 months.

Design of experiment

In addition to the standard ECRHS III evaluations, participants provided exhaled breath samples collected using the PExA method. SP-A and albumin content of exhaled samples were determined by ELISA. The obtained values were used to estimate reference intervals for the protein concentrations in particles for a Swedish population without lung disease and to investigate relationships between the observed protein concentrations, age, gender, anthropometry and spirometry data.

Outcomes

- Distribution and reference intervals for SP-A wt% concentration
- Distribution and reference intervals for albumin wt% concentration
- Associations of the observed protein concentrations with age, gender, anthropometry and spirometry data

3.3 Sampling of particles in exhaled air

3.3.1 PExA instrument

The PExA instrument is depicted and described in figure 11. In paper III the valve system (2) was removed and exhalations were made directly into the breath reservoir.

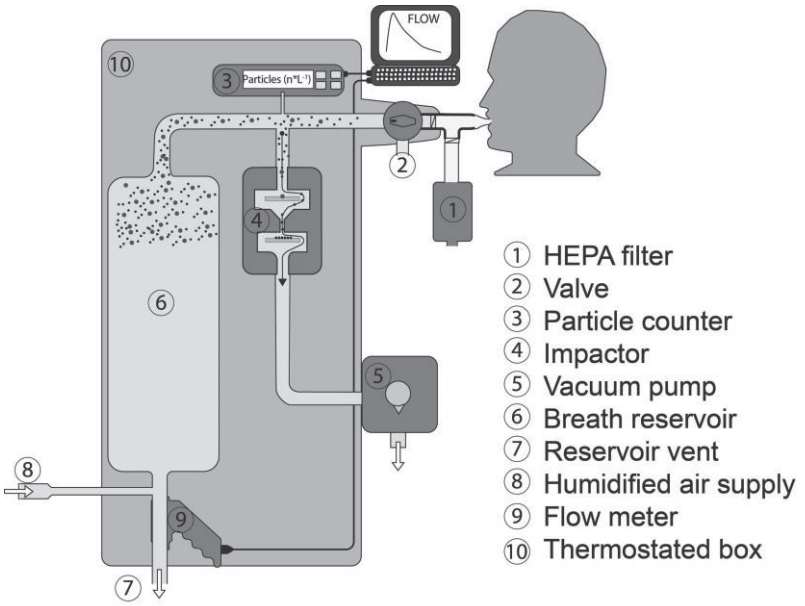

Figure 11. Particle-free air is inhaled through a HEPA-filter (1). By operating a two-way valve (2), exhalations can be directed to ambient air or to the PExA instrument for sampling. A fraction of the air is characterized by an optical particle counter (3) that operates at flow of 20 mL·s^{-1} while the remainder is drawn through the impactor. The particle counter measures the particle size and number concentrations in eight size intervals from 0.41– 4.55 μm. Particles are sampled from the aerosol with a two stage inertial impactor (4) that is set up with a constant volumetric flow of 230 mL·s^{-1} using a vacuum pump (5). The impactor 50 % cut-off is 7.0 μm for the upper stage and 0.5 μm for the lower stage. The second impactor stage samples particles between 0.5–7.0 μm on a hydrophilic PTFE-membrane surface. To handle exhalations exceeding the flow rate through the impactor, a reservoir (6) that can buffer the exhaled air is used. The reservoir is open at the bottom of the instrument (7) but is supplied with particle-free and humidified air (Respiratory Humidifier Fisher&Paykel MR 700) heated to 36 °C (8) at a flow rate of 280 mL·s^{-1}; the supplied particle free air has a slightly higher volumetric flow than what is consumed by the impactor and particle counter and the small over pressure generated prevents diffusion of ambient particles into the instrument in between exhalations. A flow meter is installed at the outlet of the breath reservoir to measure exhalation flow into the reservoir (9). All parts of the PEXA instrument, except the mouthpiece, are in a thermostated box at 36 °C (10).

3.3.2 Inertial impactor

A modified PM10 cascade impactor from Dekati (Dekati Ltd., Tampere, Finland) was used for sampling particles in the PExA instrument.

The size range of particles collected by the impactor was modified by adjusting the nozzles directly above the impaction plate and by increasing the volumetric flow rate through the impactor. The new cut-off sizes were calculated using the equation for the Stokes number (see appendix A). Impactor cut-off sizes after modifications were determined to 7.0, 2.0 and 0.5 μm in diameter. The impactor sampling efficiency can be uncertain when close to the cut-off diameter (fig 12). However, the overall sampling efficiency between upper and lower cut-off is close to 100%.

In the pilot study for paper I, the impactor was set up to sample particles from 0.5–2.0 μm. In order to increase the amount of collected material, the impactor was modified to sample particle from 0.5–7.0 μm on one impaction plate. This setting was used for all studies presented in this thesis.

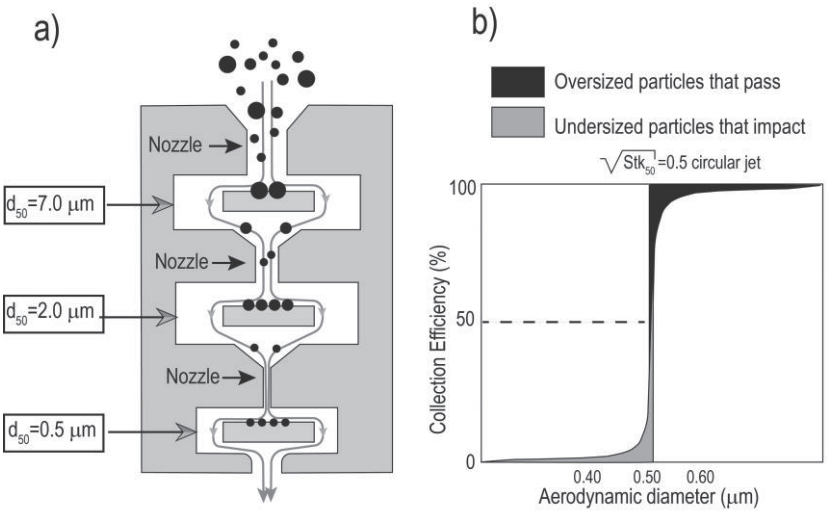

Figure 12. a) Three stage impactor with recalculated cut-off:s. b) A cut-off with a sharp but not ideal cut-off shape. For most studies the impactor was modified to sample particles between 0.5–7.0 μm by changing place of the nozzles above the 0.5 and 2.0 μm impaction stage.

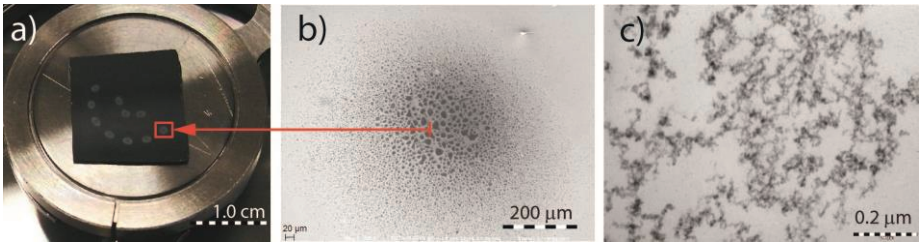

Figure 13. a) Photo of silicon surface with ten visible spots of sampled PEx. b) PEx imaged on silicon wafer by scanning electron microscope, reproduced with permission from Helene Olofson. c) Electron microscopy of PEx sampled and fixated on a hydrophilic polytetrafluoroethylene membrane. The PEx spot morphology resembles that of degraded surfactant (138). Electron microscopy was performed in collaboration with Bengt R Johansson at the Electron Microscopy Unit, Inst of Biomedicine, at University of Gothenburg.

3.3.3 Particle counting

The OPC Dust monitor 1.108 (Grimm Aerosol Technik GmbH, Ainring, Germany), was used to measure particle size and particle number concentrations in the aerosol sampled by the impactor. Based on the light scattering that results when the particle is illuminated by a laser beam, the particle size and the particle number concentration can be determined. The instrument was operated in a 1 second averaging mode, and delivered concentration data in eight size intervals (called bins). The OPC bin sizes were based on calibration with NIST-traceable polystyrene latex spheres. Exhaled particles are expected to have optical properties similar to water. Holmgren *et al.* recalculated the limits of each size bin using Mie theory to account for the difference in refractive index between polystyrene and water (101). The recalculated size bins were 0.41–0.55, 0.55–0.70, 0.70–0.92, 0.92–1.14, 1.14–1.44, 1.44–2.36, 2.36–2.98, 2.98–4.55 µm in diameter.

3.3.4 Sampled particle mass

The sampled mass of PEx can be calculated from the equation:

$$\mathbf{M}\ (\mathrm{kg}) = \mathbf{E} \cdot \mathbf{F}\ (\mathrm{L \cdot s^{-1}}) \cdot \mathbf{V}\ (\mathrm{m^3}) \cdot \mathbf{D}\ (\mathrm{kg \cdot m^{-3}}) \cdot \mathbf{T}\ (\mathrm{s}) \cdot \mathbf{C}\ (\mathrm{n \cdot L^{-1}})$$

Where: M=sampled mass by the impactor, E= impactor sampling efficiency, F= volumetric flow rate through the impactor, V: particle volume, D=particle density, T=time, C=particle number concentration

Impactor sampling efficiency (E): For particles in bin 2 to bin 8, 100% sampling efficiency was assumed. For size bin 1, it was estimated that 36% of particles were sampled. The fraction of particles sampled in bin 1 was

calculated from the fraction of particles above the impactor cut-off divided by the width of the bin.

Particle Volume (V): In bin 1 a flat particle size concentration distribution was assumed and the equation described by Nicas *et al.* (139) was used to calculate characteristic particle volume:

$$V_{characteristic} = (\pi/24) \cdot (d_{upper}^4 - d_{lower}^4) / (d_{upper} - d_{lower})$$

In bin 2 to bin 8 an exponential decrease in particle concentration was assumed. Characteristic particle volumes in bin 2 to bin 8 were calculated using the volume of sphere formula and characteristic particle diameter. In bin 2 to bin 8 the characteristic particle diameter was calculated from the following equation:

$$d_{characteristic} = d_{lower} + 0.25 \cdot (d_{upper} - d_{lower})$$

Particle density (D): The particles are mainly composed of water, lipids, proteins and around 1% saline. For simplicity the particles were assumed to have a density of 1.0 g·mL^{-1} when calculating particle mass from particle volume. In comparison, surfactant preparations from BAL were found to have a density of around 1.085 g·mL^{-1} (126) and in plasma the reported density was 1.020 g·mL^{-1} (140).

The equations to calculate sampled particle mass were:

$$M_{bin1} = 0.36 * 0.23 * (5.91 * 10^{-20}) * 1000 * 1 * \sum_{i=m}^{n} C_{bin1=i}$$

$$M_{bin2} = 1 * 0.23 * (1.06 * 10^{-19}) * 1000 * 1 * \sum_{i=m}^{n} C_{bin2=i}$$

$$M_{bin3} = 1 * 0.23 * (2.25 * 10^{-19}) * 1000 * 1 * \sum_{i=m}^{n} C_{bin3=i}$$

$$M_{bin4} = 1 * 0.23 * (4.85 * 10^{-19}) * 1000 * 1 * \sum_{i=m}^{n} C_{bin4=i}$$

$$M_{bin5} = 1 * 0.23 * (9.39 * 10^{-19}) * 1000 * 1 * \sum_{i=m}^{n} C_{bin5=i}$$

$$M_{bin6} = 1 * 0.23 * (2.44 * 10^{-18}) * 1000 * 1 * \sum_{i=m}^{n} C_{bin6=i}$$

$$M_{bin7} = 1 * 0.23 * (8.33 * 10^{-18}) * 1000 * 1 * \sum_{i=m}^{n} C_{bin7=i}$$

$$M_{bin8} = 1 * 0.23 * (2.01 * 10^{-17}) * 1000 * 1 * \sum_{i=m}^{n} C_{bin8=i}$$

With the total mass of collected particle being the sum of masses calculated for each bin

$$M_{tot} \sum_{i=1}^{8} M_{bin\_i}$$

Source of error

The OPC was operated to measure particles in the size range of 0.41–4.55 µm in diameter. This means that particles between 4.55–7.0 µm are sampled by the impactor but not measured by the OPC. This is a potential source of error when calculating sampled particle mass. Particle mass in the 4.55–7.0 µm interval is generally less than 5% of the total mass sampled and the error is assumed to be low.

3.4 Particle sampling surfaces

Different sampling surfaces were used between the studies. Initially sampling was done using in-house prepared silicon wafers. The sampling surface was later changed to a hydrophilic polytetrafluoroethylene membrane (PTFE-membrane).

Silicon sampling surface (Paper I and II)

The silicon wafers were cut into appropriate size from a large silicon wafer using a diamond glass cutter. The size of the silicon wafer was approximately 2 cm^2. The silicon wafer was cleaned in four different solvents for 15 minutes using ultra-sonication, the order of solvents used are; 1% nitric acid, heptan, aceton and 70% ethanol. To decompose and oxidise remaining organic residues the silicon was treated with 185nm UV-light for 20 minutes and just prior to use the wafers were washed in MQ-water.

Hydrophilic PTFE-membrane sampling surface (Paper III and IV)

In paper III and paper IV a hydrophilic PTFE membrane was used as a sampling surface. The high chemical inertness of PTFE makes it compatible to a wider range of extraction solvents. Generally, PTFE is highly hydrophobic and this could be problematic when sampling hydrophilic particles. If particles have low affinity for the substrate there is a high risk of "bounce off and blow off" effect inside the impactor (141). The PTFE-membrane used in paper III and IV was purchased from Millipore (FHLC02500) and was made hydrophilic by a process that is a Millipore trade secret.

The PTFE-membrane from Millipore was delivered ready to use and for each membrane there was a thin plastic support that would protect the backside of the membrane from contamination. The PTFE-membrane had a diameter of 25 mm and was compatible with the impactors system to secure the membrane, using a lock ring to press the membrane flush to the surface of the impactor plate. After extraction, the section of the membrane where particles were deposited was cut out using a clean scalpel and the sample was placed in a clean polypropylene tube.

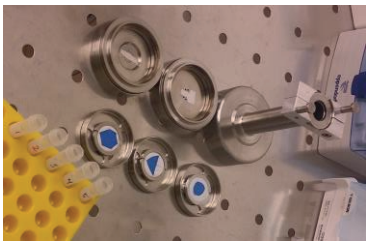

Figure 14. Photo of the three nozzle plates and the three impaction plates of the Dekati 3-stage impactor. The white PTFE-membrane and the blue plastic support can be seen on the impaction plates where the samples have been cut out.

3.5 Chemical analysis of sampled particles

3.5.1 Sample extraction

Paper I

The silicon wafer with the PEx sample was placed in a glass vial collection surface down. The PEx samples were extracted from the silicon wafer by ultra-sonication for five minutes into 315 µL of desorption solvent, prepared as 115 µL 10 mM PBS pH 7.4/ 0.15M NaCl (Medicago AB, Uppsala, Sweden)/ 0.13% Tween-20 (v/v) (Bio-Rad, Hercules, CA, USA) diluted with 200 µL

water. After extraction the eluted sample was transferred to 2 mL Protein LoBind Tubes (Eppendorf) and dried thoroughly by evaporation under reduced pressure. The dried samples were stored frozen at -20 °C before analysis.

The EBC samples were allowed to reach room temperature and centrifuged for 5 min at 200 relative centrifugal force (RCF) to collect all the liquid in the bottom of the condensation tube. The liquid was transferred to 2 mL Protein LoBind Tubes and dried by evaporation under reduced pressure. The dried down EBC samples were stored frozen at -20 °C before analysis.

On the day of the analysis the PEx samples were reconstituted with 115 µL MilliQ-water and the EBC samples were reconstituted with 115 µL of 10 mM PBS pH 7.4/ 0.15M NaCl / 0.13% Tween-20 (v/v). The final buffer composition was the same for PEx samples and EBC samples.

Paper II

Silicon wafers with sample were stored in Teflon containers (designed for microchips) for 6-36 months at -80 °C. On the day of the analysis, the silicon wafer with the PEx sample was placed face down in glass vial. To the glass vial 100 µL of extraction buffer containing 10 mM PBS pH 7.4/ 0.15 M NaCl / 0.05% Tween-20 (v/v) and 1% BSA (w/v) (Prod #:28352, 37525; Thermo Scientific, Rockford, IL, USA) was added. To cover the silicon wafers an additional 225 µL of Milli-Q water was added, resulting in final extraction volume of 325 µL. The samples were heated to 37 °C for 10 minutes using a water bath followed by 2 minutes of ultra-sonication and 60 minutes of shaking at 400 RPM in room temperature (21 °C). After extraction 125 µL (38% of the total sample) was taken for the SP-A analysis and 125 µL was taken for the albumin analysis. SP-A analysis was done the same day as the extraction. Albumin samples were stored at 4 °C overnight and analysed the following day.

Paper III

Prior to extraction, PEx samples were brought to room temperature. Internal standard, 15 pmol of PC (17:0 20:4) and PC (17:0 14:1) in methanol, was added to each sample and allowed to dry in the air for 10 min. 160 µL of extraction solvent (MeOH:chloroform:40 mM Ammonium acetate (6:3:2)) was added to each sample. To ensure that sampling membrane was at the bottom of the vial and covered by extraction solvent, samples were centrifuged for 2 min at 10 000 RCF. Samples were Vortex-mixed for 30 s and transferred to glass vials for further analysis.

Paper IV

Samples were stored dry on the PTFE-membranes in -80 °C freezers until analysis. Before analysis samples were extracted from the PTFE-membranes using 140 µL of extraction buffer containing 10 mM PBS pH 7.4/ 0.15M NaCl/ 0.05% Tween-20 (v/v) and 1% BSA (w/v) (Prod #:28352, 37525; Thermo Scientific, Rockford, IL, USA). Extraction was made for 60 min using a thermomixer (Comfort, Eppedorf) set at 37 °C and 400 RPM. The extracted sample was split into three parts, 40 µL per vial: one for SP-A analysis, one for albumin analysis and the third sample was stored as a backup (fig 15). Before the analysis the sample was diluted with assay buffer in the proportion of 1:2.

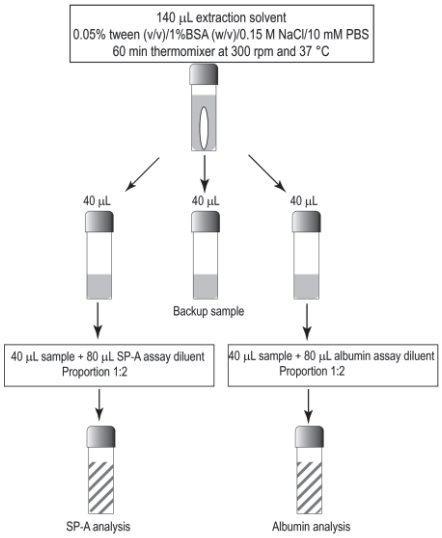

Figure 15. Extraction protocol for SP-A and albumin on PTFA-membrane.

3.5.2 ELISA

Paper I

In-house SP-A ELISA

The total volume of reconstituted EBC and PEx samples was 115 µL and 100 µL were used for the SP-A ELISA. SP-A was analysed using a sandwich ELISA protocol adapted from Ellingsen *et al.* (142). No pure SP-A protein standard of known concentration was available to use as calibration standard for the assay. A standard curve was constructed from a reference serum sample that was produced by pooling serum from 16 individuals with a known high response. This pooled serum was aliquoted and used as a standard for the in-house ELISA. The arbitrary units (au) were used for the observed SP-A

concentrations. The pooled serum used as a standard was given the concentration of 300 au. For the standard curve, the serum standard was diluted by a factor of three with 10 mM PBS to create the highest standard concentration of 100 au, this was then diluted further with PBS to create a calibration curve between 0–100 au.

The immunoassay plate (Medisorp 96-well; NUNC) was coated with the polyclonal goat antibody for human SP-A (AB3422; Millipore, Billerica, MA, USA) (10 µL antibody diluted in 11 ml PBS). The plate was incubated for 2 h at 37 °C and washed three times with 10 mM PBS / 0.05% Tween-20. The plate was blocked to unspecific binding using 0.5% (w/w) gelatin by incubation for 2 h at 37 °C. Standards and samples were added to the washed plate and the plate was incubated over night at 4°C. The following day, the plate was washed three times before incubation with the biotin-labelled monoclonal mouse antibody for human SP-A (HYB 238-04; Antibody Shop, Gentofte, Denmark) (10 µL of antibody was diluted in 11 mL PBS). After incubation with monoclonal antibody for 2 h at 37 °C the wells were washed three times. Streptavidin–HRP conjugate (Sigma-Aldrich, St Louis, MO, USA) (10 µL in 11 ml PBS) was bound to biotin during incubation for 2 h at 37 °C. The plate was washed three times and HRP substrate OPD/H_2O_2 was added for colour development. The reaction between OPD and HRP was allowed to proceed for around 10 minutes before denaturing HRP and stopping the reaction with 0.5 M H_2SO_4. Plate absorbance was read at 490 nm using the BioTek ELX-808UI (Highland Park, MI, USA) plate reader. Concentrations were calculated from a 4-parametric standard curve by the KC Junior software V1.41.8 (Highland Park, MI, USA) and reported in the arbitrary unit, au.

Albumin ELISA

The total volume of reconstituted EBC and PEx samples were 115 µL, from this 10 µL were used for the albumin ELISA. Albumin concentration was measured using a commercial ELISA from ICL (E-80AL, Immunology Consultant Laboratory, Newberg, OR, USA). The albumin samples were diluted 10 times with assay diluent and analysed according to the manufacturer's instructions.

Paper II

SP-A and albumin ELISA

SP-A was analysed with a commercial ELISA kit from Biovedor (RD191139200R, Brno, Czech Republic). The commercial assay was compared to the in-house assay used in previous study and short experimental

validation of the commercial ELISA kit for analysis of PEx was carried out prior to the analysis of clinical samples (see appendix A). Albumin was analysed with the same commercial albumin ELISA from ICL as in study I. Different protocols from Study I were used for sample preparation and dilutions., i.e., the extracted sample aliquots of 125 μL each were diluted with 100 μL of corresponding assay diluent supplied with SP-A and albumin ELISA kits. All calibration standards and controls were prepared with a buffer of the same composition as the final buffer for samples preparations. These modified dilution buffers were prepared by mixing extraction buffer and assay dilution buffers supplied with ELISA kits in the same ratio as used for the samples (125 parts extraction buffer to 100 parts dilution buffer). Samples were analysed in duplicates.

Paper IV

SP-A and albumin ELISA

SP-A was analysed with the same commercial SP-A ELISA as used in paper II. Albumin was analysed with a commercial albumin ELISA, the same assay as used in Studies I and II. The assays were performed according to the manufacturer's instructions, with minor modifications to the buffer composition and incubation times. All calibration standards and controls were prepared and assayed in the same assay buffer as particle samples. On the analysis day, samples were thawed to room temperature and diluted 3 times with provided ELISA sample diluents. The modified dilution buffer was prepared by mixing extraction buffer and manufacturer dilution buffer in the same ratio as used in the samples (1 parts extraction buffer to 2 parts assay dilution buffer). The plate incubation times was extended from 2 to 3 hours for SP-A assay and from 60 to 90 min for albumin assay. Samples were analysed with a single measurement per samples, a backup sample was available if there was reason to suspect that the analysis failed.

3.5.3 Quadrupole Mass spectrometry

Quantification of DPPC and POPC was achieved using selected reaction monitoring (SRM) on a triple quadrupole mass spectrometer (Sciex API3000, AB Sciex, Toronto, Canada) equipped with an electrospray ion source.

Samples were delivered by direct flow injection using a CTC HTS PAL autosampler (CTC Analytics, Zwingen, Switzerland) and a Shimadzu 10ADvP LC system (Shimadzu, Kyoto, Japan). An isocratic mobile phase containing methanol:chloroform: 40 mM ammonium acetate (6:3:2, v/v/v) was used for sample delivery. A flow gradient was performed starting with a flow rate of 50

µL/min for 0.1 min followed by 30 µL/min for 1.0 min and increased to 200 µL/min for 0.2 min. The mass spectrometer was operated in positive mode, with an ion source temperature set at 375°C. The ion spray voltage was set to 5000 V. The declustering potential, collision energy and cell exit potential were individually optimized for DPPC and POPC SRM assays.

The following transitions were used in SRM analysis: m/z 734.2 > 184.1 for DPPC, 760.2 > 184.1 for POPC. The transition for internal standards were 718.2 >184.1 and 796.2 > 184.1 for PC (17:0 14:1) and PC (17:0 20:4), respectively. Data analysis was performed with Analyst software 1.6.1. (AB Sciex, Toronto, Canada). DPPC and POPC were quantified using calibration curves prepared in a concentration interval between 0.005 and 0.5 µmol/L.

3.6 Clinical assessment

3.6.1 Spirometry

Spirometry was performed with Spirare SPS310 sensor and Spirare 3 software (Diagnostica AS, Oslo, Norway) in accordance ATS/ERS criteria. Percent predicted normal values were derived using the ECCS/ERS reference equations (143).

3.6.2 FENO

FENO was measured by a NIOX system (NIOX; Aerocrine AB, Stockholm, Sweden). Measurements were performed before spirometry in duplicate at three exhalation flow rates, 0.05, 0.10 and 0.27 $L \cdot s^{-1}$, referred to as $FENO_{0.05}$, $FENO_{0.10}$ and $FENO_{0.27}$. A flow rate within 10% of the target flow was acceptable. Measurements ensured a positive mouth pressure of at least 5 cm H_2O during exhalations in accordance with ATS guidelines (66).

3.6.3 Allergy test

The ImmunoCAP Phadiatop (Pharmacia & Upjohn Diagnostic, Uppsala, Sweden) test was used to screen for allergy. The test measures presence of IgE against the most common upper airway allergens (birch pollen, mugwort pollen, timothy pollen, mite, cat, dog, horse, mould) in blood. The test has been found to have good diagnostic accuracy for screening for allergy with sensitivity and specificity above 70% (144).

3.6.4 Birch pollen measurement

Airborne pollen was collected using a 7-day recording volumetric spore sampler (Burkard Scientific Ltd, Uxbridge, Middx, UK). Air was drawn

through the sampler and pollen/spore grains were deposited on sticky tape inside the sampled. The spore capturing tape had a movement that was synchronized to a clock and time was derived from the position on the tape. The pollen grains that were captured on the tape were counted and classified manually.

3.7 Statistical methods

All statistical analysis was performed using the SAS software package (version 9.2 and 9.4) except paper IV, where IBM SPSS 22.0 was used for data analysis. All tests were two sided and the null hypothesis was rejected for p-values of < 0.05.

3.7.1 Samples with low concentrations

The large difference in emitted amount of particles by different individuals resulted in a large range of concentrations during the chemical analysis when sampling was based on the exhaled volume of breath instead of a fixed amount of particles. Samples of high amount can be diluted whereas samples with a low amounts had to be handled in different ways. There are several approaches for samples of low concentrations: use measured but uncertain values, give the samples a fixed estimated concentration or exclude the samples. The preferable method varies with chemical method used, how LoQ is defined and how the data will be used. Due to the complexity in handling low and uncertain values statisticians were consulted to maximize power for the statistical tests while minimize bias in the results. Definitions often used to describe samples with concentrations too low to measure accurately are:

- LoB: Limit of blank is the signal that is generated by blank samples often calculated as $LoB = mean_{blank} + 1.64(SD_{blank})$ (145)
- LoD: The limit of detection (LoD) is generally taken as a signal that is different than a blank sample with a high degree of confidence but the methods to determine this varies. The mean value of the blank plus three standard deviations is commonly used as a simple definition (146). A more accurate definition is based on the limit of the blank LoB and the standard deviation (SD) of samples with a low concentration, using the formula $LoD = LoB + 1.65 \cdot (SD_{sample})$.
- LoQ: The limit of quantification is often a more useful parameter than LoB and LoD as this value is often used to make decisions whether to reject or accept a result for a given

application. LoQ has no simple predefined definition and acceptable performance targets have to be chosen based on the application. A simple approach for estimating LoQ is to use the raw data from the standards and back calculate the concentration from the standard curve. From the back calculated concentrations the accuracy (true/back calculated value) and the coefficient of variation (CV) is determined for each concentration level and used for finding the acceptable assay range. A more reliable method is to use samples over a wide range of concentrations and construct performance profiles with estimates and confidence limits. Performance parameters that are often used to construct performance profiles are CV and relative error (RE). These plots can be very useful for finding the upper and lower concentration range that have acceptable performance for the indented use of the results. Profile plots with CV% and RE% are shown for SP-A ELISA in appendix A.

3.7.2 Paper I

The value for each observation was presented and median and interquartile range was used for summary statistics. Due to the many values below the quantification limit no statistical analysis was done with the EBC data. Association between sampled particle mass to sampled SP-A and albumin was evaluated by Spearman's rank correlation coefficient and for this correlation the means from first and second sampling session was used. Using the means from first and second sampling session, association between SP-A in exhaled particles and SP-A in blood was assessed using Spearman's rank correlation coefficient. For PEx samples and serum samples the within and between subject CV was determined. The intra-individual CV was calculated as the mean of each individual's CV, that was calculated from session one and two. Only individuals with both measurements above LoQ were included in this calculation. The inter-individual CV was calculated from each individual mean concentration from which the overall standard deviation and mean was calculated. Samples below LoQ were assigned the value of LoQ/$\sqrt{2}$ (147).

3.7.3 Paper II

Samples from each individual were grouped together during the ELISA analysis to minimize analysis variability within these samples. To avoid systematic differences between the two groups, controls and individuals with asthma were alternated and evenly distributed on the 96-well plates. The outcome variables were presented with median values together with first and

third quartile. The exact Wilcoxon-Mann Whitney U test was used to compare groups. The Wilcoxon signed rank test was used to test differences between seasons for paired observations. The assumption of similar distribution between groups for the exact Wilcoxon-Mann Whitney U test and the assumption of symmetric distribution of the differences around the median for the Wilcoxon signed rank sum tests were evaluated by histogram plots. For the Wilcoxon signed rank sum tests a log transformation was used in cases where this improved the symmetry of the data around the median inter-seasonal difference as described by Altman *et al.* (148). Spearman's rank correlation coefficient was used to assess inter-seasonal change in exhaled PEx mass per litre of breath and inter-seasonal change in FENO. Relationship between exhaled particle mass and exhaled SP-A and albumin mass was determined by linear regression and the coefficient of determination was presented with the p-value. For the linear regression analysis, data from both seasons and data from both the asthma and control group were combined in the analysis. The decision to combine the groups was based on the large overlap between the groups in the scatter plot.

3.7.4 Paper III

The data were analysed using a mixed model employing restricted maximum likelihood with type of manoeuvre as the fixed effect and individual as the random effect. By using a mixed model, it was possible to get a good estimate on the effect of breathing manoeuvre despite the large variability seen between subjects. The variables, PEx, DPPC and POPC mass per litre of breath did not have a normal distribution but after log transformation the distribution was approximately normal. Therefore, they were log transformed for the statistical analysis and back transformed by exponentiation after the analysis. Because of the logarithm rules, e.g. log(a)-log(b)=log(a/b), the difference between manoeuvres takes the form of ratios after being back transformed to the original scale. For variables that were log transformed, the difference between manoeuvres was estimated as a relative effect. The concentrations of DPPC and POPC in PEx had an approximatively normal distribution and were not log transformed. The difference between manoeuvres for concentrations of DPPC and POPC in PEx was therefore expressed as an absolute difference in concentration. The assumptions of normal distribution and homogeneity of variance for the models were tested by histogram plots of residuals and plots of residual *vs*. fitted value and were determined to be acceptable. When calculating the concentration of DPPC and POPC in PEx, samples with a very low mass of sampled PEx and a concentration below LoQ in the chemical analyses were excluded.

3.7.5 Paper IV

The variables, SP-A and albumin expressed as wt% of PEx, were not normally distributed and were log transformed with natural logarithm. The log transformed data showed close to normal distribution for both variables and were used for statistical data evaluation.

Reference intervals were chosen to be expressed as 90%RI. The 90%RI were calculated from the log transformed data as mean ± 1.64·SD. These values were then exponentiated to get RI in the original scale.

Multivariable linear regression analyses were used to investigate relationships between the observed protein concentrations and potential predictors: age, gender, body size, smoking status, spirometry data, atopy and exhaled particle production. Spearman correlation was used to estimate collinearity between predictors. Only one predictor from the group of the collinear predictors was allowed for the statistical modelling. Predictors selection was based on the univariate linear regression analysis, with all predictors with p-value < 0.25 being initially included into the multivariable linear regression analysis. A stepwise elimination of the predictors was applied to select the best fitting model. The model validity was checked by the residual distribution evaluation. Only models that fulfilled requirements of normal distribution for the residuals and their independence of the predicted values were accepted.

4 RESULTS AND DISCUSSION

4.1 Paper I

4.1.1 Pilot study

The sampled amount of SP-A was much higher for the PEx samples than for the EBC samples (table 2). For PEx samples there was a clear association between collected PEx mass and the amount of SP-A and albumin (fig 16). There were two outliers among the PEx albumin data. This may have been a result of contamination from the serum samples that were run at the same time (data not presented). No association between albumin in PEx and albumin in serum was observed, thus analysis of albumin in serum was excluded from the final study protocol.

Table 2. SP-A and albumin in PEx compared to SP-A in EBC

	PEx-SP-A (au)		PEx-Albumin (ng)		EBC-SP-A (au)	
Individual	Session 1	Session 2	Session 1	Session 2	Session 1	Session 2
1	1.90	2.98	4.5	18.0	<LoQ	<LoQ
2	1.07	0.72	7.2	<LoQ	<LoQ	0.23
3	0.78	0.74	<LoQ	55.2	<LoQ	<LoQ
4	2.60	1.93	5.4	8.5	<LoQ	<LoQ
5	0.63	0.35	<LoQ	<LoQ	<LoQ	1.60

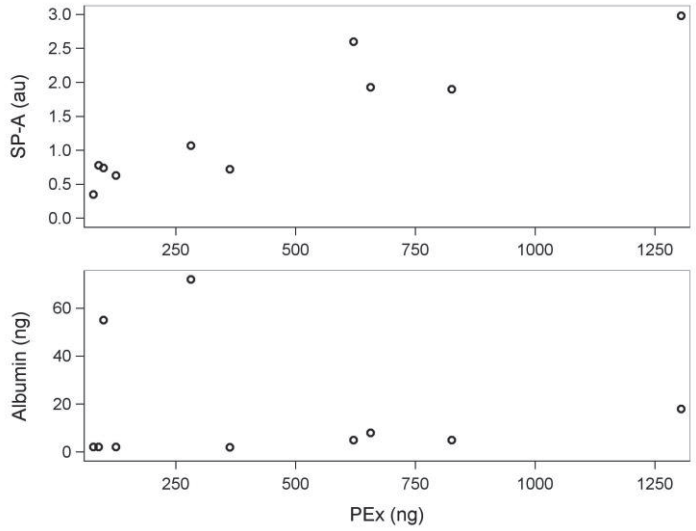

Figure 16. Relationship between sampled PEx mass and sampled protein mass. Five individuals with two measurements per individual.

4.1.2 Paper I

SP-A was detected in all samples collected with the PExA method. Even though only 10% of the sampled material was used for the albumin analysis, albumin was detected in 72% (13/18) of the PEx samples. For the same volume of exhaled air collected using the EBC method, SP-A was detected only in 22% (4/18) of the samples and albumin in 5% (1/18) of the samples.

The two methods use different principles to sample the proteins from breath. In the PExA method, particles are sampled by impaction. Exhaled breath condensate (EBC) is collected by cooling the exhaled breath and collecting the condensate. The only way to transport non-volatiles in breath is as droplets/particles that follow the air stream during exhalation (74). During exhaled breath condensate sampling the particles grow by condensation in the cooling chamber, and some particles deposit by sedimentation or impaction. Condensation is not an efficient method for sampling particles and it is not possible to predict the sampling efficiency or dilution factor for the sampled particles (149). The volume of condensate is mainly determined by the duration of the sampling, the volume of exhaled breath (150) and the chemical composition of the particles (76), and is not directly related to the sampling rate of particles.

Another difference between the two methods is the use of different breathing manoeuvres. In the PExA method, an airway closure and reopening manoeuvre is used to increase particle formation in small airways. It has been recommended that EBC is sampled with tidal breathing and this is what the instrument is designed for (3). To test if the large difference in sampled protein amount between the two methods could be explained by the use of different breathing manoeuvres, three EBC samples were collected with the airway opening manoeuvre. The sampled amount of SP-A was below LoQ for all three samples, indicating that the difference between the two methods was likely not a result of breathing manoeuvre alone. In one of the individuals the EBC amount (condensate volume) was reduced to half with the airway opening manoeuvre but in the other two the change was less than <10%. In the EBC instrument the air is exhaled through a condensation chamber and the transit time is decreased with increasing air velocity. Thus, the increase in exhalation flow by the airway opening manoeuvre, compared to tidal breathing, may decrease the sampled EBC volume. It would have been optimal to let more subjects using the same breathing maneuver as used for PEx sampling.

The surface of the EBC collection tube was different than the silicon wafer surface used for PEx sampling, which may affect the sampling. The condenser coating has been found to influence the sampling efficiency of EBC methods for different compounds (76).

For the PExA method, a strong correlation between sampled PEx mass to SP-A and albumin mass was observed, r_s=0.93 ($p<0.001$) and r_s=0.86 ($p=0.003$) respectively. Since non-volatiles are transported as particles in breath a strong correlation was expected. The mean intra-individual CV was 13% for SP-A and 50% for albumin. The inter-individual CV was 25% for SP-A and 104% for albumin. No adjustment for the sampled PEx mass was done, if SP-A amount would have been normalized for sampled PEx mass the CV would likely have been lower.

The intra-individual and inter-individual CV for SP-A in serum was 7% and 127% respectively. No correlation between the SP-A serum concentrations and PEx concentrations was observed, demonstrating that SP-A in serum and SP-A in PEx are determined by different factors.

This was the first study where SP-A concentrations in PEx were measured. A limitation with the study was the in-house developed ELISA. In this ELISA, pooled serum was used to construct a standard curve. The serum most likely contained some cross-reacting component or heterophilic antibody (see

appendix A). Thus, the measured sample concentrations were only semi-quantitative.

The measured SP-A and albumin amounts were not adjusted for the different mass of sampled PEx. This could have been done by using a regression model.

4.2 Paper II

The main finding in paper II was that there was a significant decline in the exhaled PEx mass per litre of exhaled breath during pollen season for the individuals with asthma and birch pollen allergy (p-value < 0.01). A reduced extent of airway closure and reopening during breathing is one possible explanation for the result. In addition, altered airway geometry and higher bronchomotor tone, resulting in airway narrowing, are likely to increase the deposition of particles during exhalation (151).

Even low levels of pollen exposure has been associated to inflammation in subjects with mild asthma and allergy (152, 153). Inflammation induced by allergen exposures have been associated to swelling of the airways, leakage of plasma proteins into the airways (44, 45) and accumulation of liquid (94). This can result in blockage of small airways and reduce the number of airways that close and reopen during the applied breathing manoeuvre. Surfactant in BAL, retrieved from allergen exposed lung segments, has been shown to have a reduced capability to maintain airway patency compared to surfactant retrieved from unexposed segments (45, 154), this was suggested to be a result of plasma protein leakage that disrupted the surfactant function.

The main hypothesis in paper II was that SP-A concentration in PEx should be decreased and albumin concentrations would be increased, as a result of inflammation, in individuals with asthma and allergy during pollen season. However, neither any significant inter-seasonal differences in SP-A or albumin concentration in PEx was observed, nor any differences between subjects with asthma and healthy controls.

Most of the subject with asthma and allergy had mild disease and normal lung function and the response to bronchodilation during pollen-season was small. The increase in FENO among the asthma subjects during pollen-season was also very modest (mean 1.3 ppb), and no significant correlation between change in albumin or SP-A in PEx and change in FENO was found. The individuals in the asthma group had mild asthma and possibly only low level airway inflammation which may explain why no significant effect on SP-A or albumin concentration in PEx was observed. A second concern is whether

small airways were affected? Pollen grains are too large to efficiently penetrate to the small airways. However micro-particles containing allergen as small as 30 nm can form when pollen grains burst in a humid environment. These can be inhaled and possibly reach the small airways (155). It is conceivable that the degree of inflammation may have been more pronounced in proximal airways, whereas PEx is likely to originate from the small airways.

Vascular leakage and increasing albumin concentration in the airways have been suggested as pathophysiological features of early asthma deterioration (44). It is assumed that there is a large alveolar-capillary concentration gradient that is disrupted during inflammation. The difference in concentration of albumin between alveolar and capillary compartments are not known, and the normal range for the albumin concentration in small airway RTLF is not established. The permeability of albumin could vary for different types of exposure that induce different degrees of permeability.

Albumin concentration in PEx samples was estimated to be around 4.1 wt% (4.1 $g \cdot dL^{-1}$). This is similar to the concentration of albumin in blood 4.5 $g \cdot dL^{-1}$ (120). Albumin is semi-permeable over the alveolar-capillary barrier and may enter the airway lumen also under normal conditions (7). Thus, albumin may not be a sensitive marker of increased plasma leakage (156). Large proteins such as fibrinogen are normally found at very low concentrations in the airways and may be more sensitive for measuring increased alveolar-capillary permeability (124, 156).

The accuracy of the calculated PEx wt% concentrations have not been validated, by direct measurements. It is therefore difficult to make a direct comparison between blood and PEx concentrations. The PEx result in our study, indicates a lower albumin concentration gradient than reported by other methods. Micro-sampling probes have indicated an airway concentration that is around 10% of the blood concentration (157), similar results have been reported in BAL studies (158). However, those methods have a high uncertainty regarding the dilution and it is also difficult to know the exact airway generation that is sampled. How albumin concentration differs between airway generations is not well known.

In subjects with asthma and allergy there was a trend for the albumin concentration in PEx to decrease during pollen season, with a median decrease of 0.5 wt% units (n=9, p=0.1) (fig 17). This change was in the opposite direction to the hypothesis of passive leakage. An alternative hypothesis to passive diffusion of albumin would be active regulation. Albumin is an important molecule for regulating the fluid balance of the airways (7) and is

responsible for around 80% of the oncotic pressure in plasma (120). Albumin may be actively lowered in the airways as a response to accumulation of fluid as this could facilitate the removal of excess fluid. Albumin may be suitable for this role, since it is a relatively weak inhibitor of surfactant, compared with e.g. fibrinogen (123, 154), and only semi-permeable over the alveolar-capillary barrier.

The main limitations of the study were the limited number of subjects included, due to technical problems during the albumin ELISA some samples were lost and albumin results were only available for 9 out of the 13 subjects included in the study. Another limitation was the uncertain exposure level of birch pollen and the difficulty to ascertain that changes in PEx measurements were associated to small airway inflammation.

The present study was a very small study with low power but important for evaluating the methods before a larger study. From the results in paper II it is possible to make a samples size estimate for a larger follow up study. From the power plot, shown in fig 18, it can be estimated that a follow up study of around 20-30 pairs per group is likely to detect a change in albumin of around 0.5 wt%.

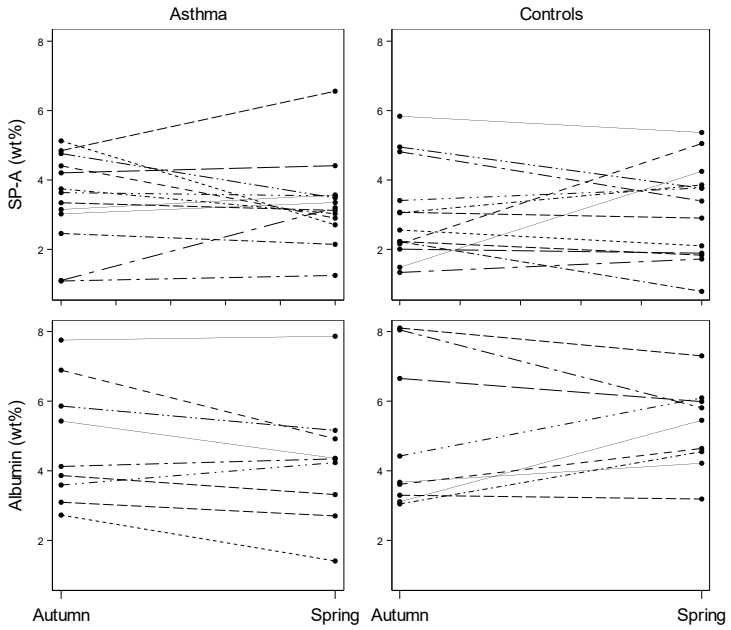

Figure 17. Inter-seasonal differences in SP-A and albumin concentration in PEx.

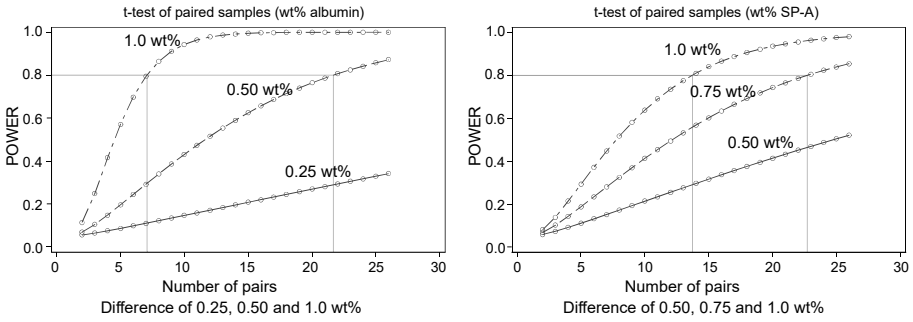

Figure 18. Sample size estimate for a larger follow up study based on the results in paper II.

4.3 Paper III

4.3.1 Pilot study

Exhaled PEx mass concentration by the forced manoeuvre was 2.2 times that of the reference manoeuvre (table 3). Exhaled PEx mass concentration by the airway opening manoeuvre was 9.5 times that of the reference manoeuvre. The hypothesis was that the dynamic airway compression would increase particle formation by the flow-mucus mechanism during a maximal forced exhalation. The increase in exhaled PEx mass with forced exhalations was small compared to the increase observed with the airway opening manoeuvre. All samples collected with the airway opening manoeuvre contained higher SP-A mass than the samples collected with the forced exhalation, indicating a lower concentration of SP-A in particles exhaled with the forced exhalation. From fig 19 it can be observed that for samples with a PEx mass below 20 ng, the ratio of SP-A to PEx becomes very unreliable as the value starts to approach a fixed background level rather than having a linear decline towards origo. When both sampled SP-A mass and sampled PEx mass are low it is difficult to draw any conclusion of the wt% concentration of SP-A in PEx as both the dominator and the numerator are uncertain and such calculations should be avoided. A blank sample should have been included in the measurement to estimate the background signal from a sampling membrane during the analysis.

Table 3. Mass concentration of PEx in exhaled breath

	Mass exhaled per litre of breath	Times that of the reference manoeuvre	
	PEx (pg·L^{-1})	PEx	
Manoeuvre	GM (95% CI)	GM ratio (95% CI$^{\#}$)	p-value$^{\#}$
Reference	190 (120–300)	N/A	N/A
Forced	410 (270–640)	2.2 (1.4–3.2)	<0.01
Airway opening	1840 (1190–2850)	9.5 (6.4–14.2)	<0.01

Measured concentrations are presented as geometric means with 95% confidence intervals in the left part of the table. The change times that of the FRC-reference manoeuvre with 95% confidence intervals are presented on the right part of the table. Results were calculated from a mixed model with type of manoeuvre as fixed effect and individual as random effect. Significant p-values are in bold text. **#**: adjusted for multiple testing using Tukey's method; GM: geometric mean; CI: confidence interval; N/A, not applicable; pg·L^{-1}: picogram per litre exhaled breath.

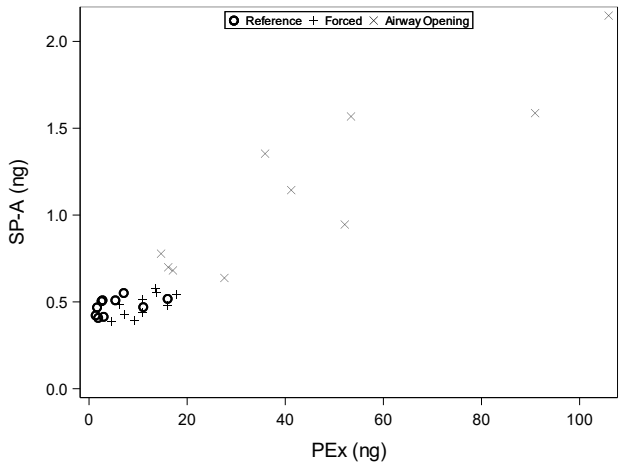

Figure 19. Plot of sampled SP-A mass as a function of sampled PEx mass

Conclusions from the pilot study

The main observation in the pilot study was that the exhaled mass of PEx when using forced exhalations was lower than was anticipated. The low amount of sampled PEx made analysis of SP-A difficult resulting in unreliable

measurements. This raised questions regarding selected methodology and study design.

Current instrument design might not be optimal for analysis of forced exhalations. The flow resistance of the PExA instrument might result in reduction of maximal flow reached with forced exhalation. If the maximal expiratory flow is determined by the PExA instrument, and not the airway physiology, the extent of airway compression is unknown. Particles might have been deposited by inertial impaction in the mouthpiece when maximal forced exhalations were used. The SP-A method was not sensitive enough for measuring PEx samples collected with ten forced exhalations and a more sensitive method was required. These questions were addressed before paper III and are described in appendix A.

The results of exhaled PEx mass concentrations in paper III after the modifications to the method were similar to the results in the pilot study. Thus, the changes made to the breathing manoeuvres and the PExA instrument between the studies did not appear to have had a large impact on the results.

4.3.2 Paper III

The aim of the study was to compare particles formed during high expiratory flows to particles formed during an airway closure and reopening manoeuvre. High expiratory flows create high air velocities that may result in shear forces strong enough to create a two-phase mist flow of gas and liquid (88). The shear forces are likely highest at the choke point where the lung cross section is lowest and the air velocity is highest. In normal individuals the choke point is located in around airway generation 3 but may progress towards airway generation 6 at the end of expiration (88). During an airway closure and reopening manoeuvre particles are formed during inhalation when closed airways reopen, i.e. the RTLF film burst mechanism. These particles are expected to form in small airways with a diameter of less than 1 mm, i.e. in terminal bronchioles (23). These two mechanisms, two-phase mist flow of gas-liquid and RTLF film burst, are expected to generate particles in very different airway regions, i.e. the small airways and the proximal airways. Particles generated during a forced exhalation are expected to have a composition reflecting the proximal airways whereas particles generated by an airway opening manoeuvre are expected to have a composition reflecting the small airways. In this study, we aimed to assess the extent of particle formation by a forced exhalation and an airway closure and reopening manoeuvre compared to a baseline manoeuvre. We also compared the DPPC and POPC composition between the particles. It is expected that the DPPC concentration

is highest in the alveoli and declines towards the glottis. The results are summarised in fig 20.

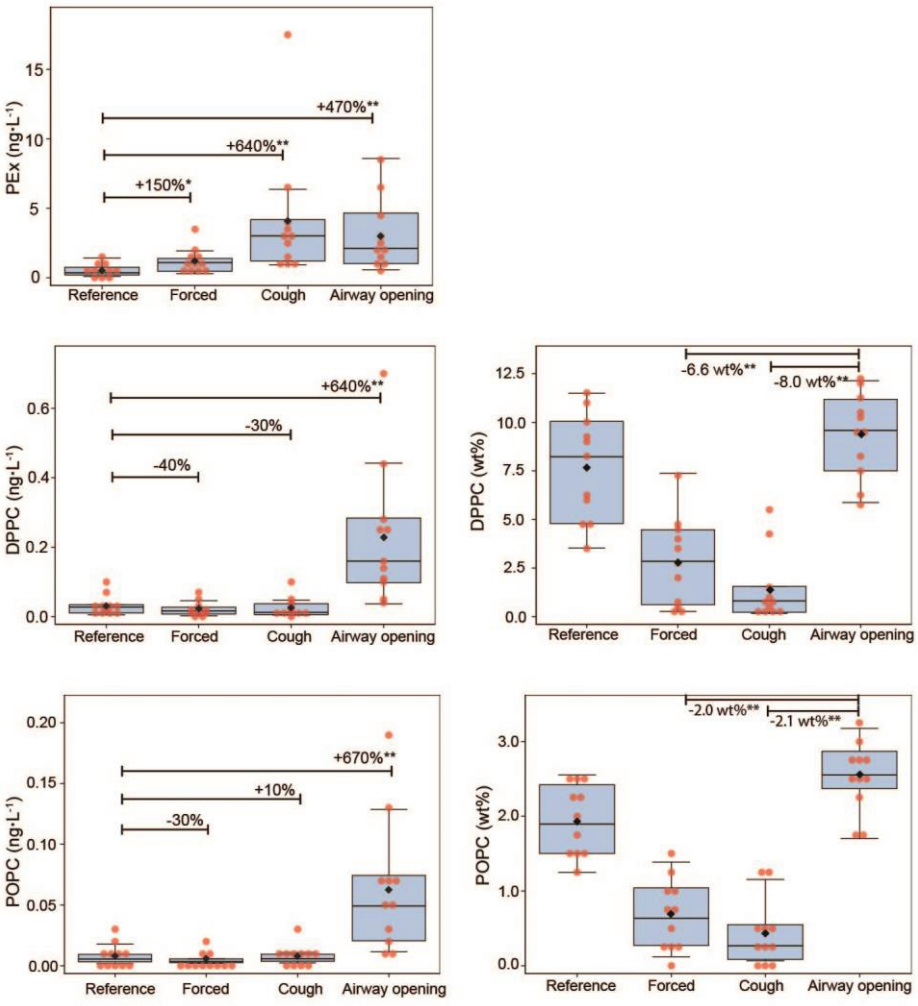

*Figure 20. Exhaled PEx mass per litre of breath. b) exhaled DPPC mass per litre of breath. c)DPPC wt% concentration in PEx. d)exhaled POPC mass per litre of breath. d) POPC wt% concentration in PEx. *: p-values<0.05; **p-value<0.01*

FRC-reference

This manoeuvre generated the lowest PEx mass concentrations and was intended as a baseline measurement. The particles exhaled by the FRC-reference manoeuvre had a DPPC and POPC concentration that was similar to the RV-reference manoeuvre. This indicated that the particles were formed during the inhalation from FRC to TLC rather than during the exhalation from TLC to RV. This suggests, that some airways are closed at FRC and are reopened during inhalation to TLC. In support of this, studies have shown that particle emission increases with ventilation volume (107).

Forced exhalation

The increase in exhaled PEx mass concentration by using a forced exhalation compared to the FRC-reference manoeuvre was estimated to be 150% (95%CI 10–470). No increase in DPPC or POPC concentration was associated with this increase in exhaled PEx mass. Thus, the additional particles generated compared to the FRC-reference manoeuvre made an insignificant contribution to the exhaled mass of DPPC and POPC.

A forced exhalation takes significantly less time to perform than a slow exhalation and this likely reduces particle deposition by gravitational settling. However, deposition by impaction in the tracheobronchial region is expected to increase with high expiratory flows and air velocities over 120 $m \cdot s^{-1}$ have been measured in trachea (88, 92). The low content of DPPC and POPC in particles formed during maximal forced exhalation suggests that these particles were likely generated in proximal or upper airways during exhalation. A study by Johnson *et al.* found that for particles smaller than 2 μm, the fraction of particles deposited by impaction was likely low during rapid exhalations (97). The fraction of large particles (2.98–4.55 μm) was increased when forced exhalations were used and was positively correlated to subjects' PEF measurements ($r_s = 0.94$, p-value < 0.01).

Cough

Cough generated the highest PEx mass concentration of all manoeuvres, 640% (95%CI 230–1570) more than the FRC-reference manoeuvre. Based on the available literature, particle formation mechanism during cough are similar to those suggested for forced exhalations, i.e. shear forces between gas phase and liquid phase that results in a two-phase mist flow (88, 89, 159). In this study we observed a large difference in particle emission between forced exhalation and cough. Due to the flow limitation caused by dynamic airway compression, cough is unlikely to generate significantly higher air velocities (88). This suggests that additional mechanisms for particle formation are activated during cough and these mechanisms are not fully understood.

Despite the large increase in exhaled PEx mass concentrations no significant increase in exhaled DPPC or POPC mass concentrations from the FRC-reference manoeuvre was observed. This suggests that the particles were formed in proximal or upper airways.

RV-reference (airway closure and reopening)

The increase in exhaled PEx mass concentration by the RV-reference manoeuvre compared to a slow exhalation was estimated to 470% (95%CI 150–1190). The increase in exhaled PEx mass concentration produced a proportional increase in exhaled DPPC and POPC mass. PEx sampled with the RV-reference manoeuvre contained the highest concentration of DPPC and POPC. This is consistent with particle formation associated to RTLF film burst in small airways during inhalation.

Analytical aspects

The background emission of DPPC and POPC, measured with the FRC-reference manoeuvre, was relatively low and neither the forced, cough nor the RV-reference manoeuvres were adjusted for baseline values. The sensitivity of the DPPC and POPC analysis was high, with LoQ:s of 117 pg and 122 pg (mass on membrane) for DPPC and POPC respectively. With this sensitivity it should be possible to measure DPPC from around 2 ng of sampled PEx, assuming a DPPC concentration of 10 wt%. Around 5 ng of PEx would be required for measuring the POPC mass, assuming a concentration of 2.5 wt%.

The wt% concentration of DPPC and POPC in PEx is a ratio and this can create biased results when values start to approach zero. If a fixed value (between 0 and LoQ) for POPC (numerator) is used and the PEx mass (denominator) approaches zero, the ratio will increase exponentially as the PEx mass decreases (fig 21). With decreasing PEx mass the uncertainty in the estimated wt% concentration becomes larger. However, if the minimum mass is set to a high value many samples will be excluded and statistical power will be lower. The chosen method for finding the minimum PEx mass was a balance between accuracy in the estimated wt% concentration and statistical power.

The minimum amount of PEx required was determined by ordering the samples from lowest PEx mass to highest and finding the minimum PEx mass required for a measurable POPC concentration. The lowest PEx mass required to have a measurable POPC concentration was 9.1 ng. Thus, samples with less than 9.1 ng amount of PEx were excluded from the statistical analysis of POPC wt% in PEx. Seven POPC samples out of the 44 samples were excluded based on the criteria of low PEx mass. Remaining samples below LoQ were given a

sampled POPC mass of 86 pg. This mass was calculated by using the concentration of LoQ/√2 (147).

For DPPC wt% calculations no sample was excluded as the lowest PEx mass was 4 ng and this sample had a measurable DPPC concentration. Samples below LoQ were given a sampled POPC mass of 83 pg.

A limitation with the study was that no marker specific for central or upper airways was measured. To get a more detailed knowledge of the region where the particles were formed, markers specific for different airway regions should have been included. The study only included healthy individuals and it is possible that individuals with hypersecretion and increased RTLF thickness would emit more particles during forced exhalation. When using the airway closure and reopening manoeuvre, very few particles are larger than the measured size range. However, during forced exhalation and cough, additional modes of much larger particles could be formed. This has been shown in previous studies (103). These very large particles are likely of an upper airway origin.

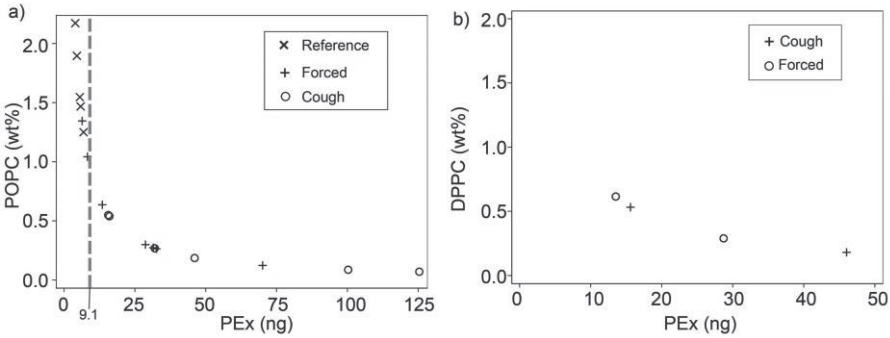

Figure 21. a) Plot of POPC wt% for values below LoQ as a function of PEx mass. The minimum PEx mass required for estimation of POPC wt% was set to 9.1 ng and is shown as a dashed line. b) Plot of DPPC wt% for values below LoQ as a function of PEx mass.

4.4 Paper IV

Reference intervals are important for finding individuals with abnormal values. These individuals may provide information on factors that have a large influence on the results for the measured variable, e.g. disease. They may also be used in clinical trials as a guide to setting inclusion / exclusion criteria.

Participants for the study were recruited from the Gothenburg area by sending out questionnaires. Out of the 342 individuals that responded to the invitation, 74 men and 83 women aged between 41–66 years without known lung disease (asthma, COPD, emphysema, bronchitis) and normal lung function were recruited to the study. The characteristics for the study population are presented in table 5. For this population, reference intervals for SP-A and albumin concentration in PEx were calculated. The purpose of these reference intervals was to improve future studies of inflammatory airway diseases.

The 90% reference intervals for SP-A and albumin concentration in PEx were calculated to 1.9–5.3 wt% and 3.6–11.2 wt%, respectively (table 4). Albumin concentration in PEx increased with 0.8% per unit increase in exhaled PEx ($ng \cdot exh^{-1}$) concentration and with 0.7% per increase in age year. The small percentage change per year can become relatively large if the age difference between groups is large. SP-A concentration in PEx was not associated with age, gender, anthropometry, atopy or exhaled PEx mass concentrations.

$$Albumin_{(wt\%)} = \exp\,(1.513 + 0.007 \cdot AGE_{(years)} - 0.102 \cdot atopy + 0.008 \cdot PEx_{(ng/exh)}$$

$$\text{Adjusted } R^2 = 0.065,\ \text{RSD: ln (Albumin)} = 0.32607:\ \text{p-value} < 0.05$$

A final protocol for sampling and measuring should be established before determining the relevant reference intervals. If changes are made to the protocols, the previously calculated interval may have to be adjusted. The final protocol should include instrumental setup, breathing manoeuvres and sample analysis. The study should be large enough so that the uncertainty in the calculated reference interval is small, at least 100 individuals are recommended (148).

The protocol for extracting and analysing samples in study IV is the one currently used. However, the exhalation flow of the breathing manoeuvre has been increased from a slow exhalation with a max flow of 600 $mL \cdot s^{-1}$ to a slightly faster exhalation, with peak flow of 1500 $mL \cdot s^{-1}$. The reason for increasing exhalation flow was to collect more material in a shorter time. In paper IV, slow exhalation flows were used to ascertain that particles were

sampled from small airways with little contribution from particles formed in more proximal airways. From the results in paper III, particle formation in central airways was found to be a small source of particle formation and it was considered safe to increase exhalation flows. The effect of increased exhalation flow on the reference intervals will be assessed in future studies. External validity of the reference interval will have to be tested in a new dataset.

The calculated RI:s for SP-A and albumin in PEx could be compared to the control group in paper II. The control group average of the autumn and spring values was used for the comparison. The median for SP-A wt% in paper II was similar to the median in the reference population in paper IV. However, the median for albumin wt% was somewhat lower in paper II than in paper IV. In paper II, the median (Q1, Q3) for SP-A and albumin wt% in PEx was 3.0 (2.0, 3.6: n=13) and 4.3 (3.9, 6.3: n=9), respectively. This may indicate that the different exhalation flow, the different sampling surfaces and the different extraction protocols may have influenced the results. However, paper II was a very small study and it is difficult to know if there was a significant difference between the used protocols. The mean age in the groups without lung diseases was similar in the two cohorts (48 and 53 years).

A limitation with reference intervals is, that it is difficult to know if the population from which the intervals were calculated is representative of the population that one intends to use them on. This makes the selection of participants crucial and it is important to include a large number of individuals.

Table 4. Exhaled particle data

PEx variable	Median (Q1, Q3)
PEx ($ng \cdot L^{-1}$)	1.8 (1.1, 3.1)
PEx ($ng \cdot exh^{-1}$)	5.8 (3.6, 8.9)
SP-A (wt%)	3.2 (2.6, 4.0)
Albumin (wt%)	6.4 (5.3, 7.8)
Data are presented as medians with first and third quartiles (Q1, Q3). $ng \cdot L^{-1}$: nanogram PEx per litre exhaled; $ng \cdot exh^{-1}$: nano gram PEx per exhalation	

Table 5. Subjects characteristics

Male/Female	74/83
Age (years), mean (range)	53.3 (40.5–66.1)
Height (cm)	172.9 ± 10.4
Weight (kg)	80.8 ± 15.2
BMI	26.9 ± 3.9
FVC % pred *	96.8 ± 12.4
FEV_1 % pred *	95.8 ± 13.5
FEV1/FVC % pred *	98.8 ± 5.5
Ex/Never-smoker (n) †	74/80
Non-atopic (n) ‡	93/62

Data are presented as mean ± SD unless stated otherwise.
BMI: body mass index; FVC: forced vital capacity; FEV1: forced vital capacity in 1 second; pred: predicted.
* Presented FVC and FEV1 correspond to the values prior to bronchodilatation.
† 3 individuals missed to report if they were never- or ex-smokers.
‡ Skin prick test was missing for two participants.

5 GENERAL DISCUSSION

5.1.1 Sampling of respiratory tract lining fluid

Common methods for sampling of RTLF are the invasive method of BAL, the semi invasive method of induced sputum sampling and the non-invasive method EBC.

All three methods have problems with unknown and variable dilutions and uncertainties regarding the airway region specificity. Despite considerable efforts to find suitable methods to adjust for the variable dilution of retrieved material no generally accepted method has been found (52). For cell counts the problem of variable dilution has been avoided by using proportions between different cells rather than the absolute cell counts. For the acellular components it has been difficult to find ratios that are reproducible.

The PExA method offers an alternative solution to the collection of material from the airways, which is non-invasive and addresses both the problem of unknown sample dilution and unknown sample origin. This is achieved by addressing three key factors that determines the concentration of analytes in the PEx sample: *PEx origin*, *PEx sampling* and *PEx analysis*. Reproducible measurements of RTLF composition require that all three aspects are reproducible and they will be discussed in details here. To achieve reproducible sampling of RTLF we need:

i) To understand how and where exhaled particles are formed.
ii) To efficiently sample the exhaled particles and quantify the sampled amount.
iii) To have sensitive and reproducible assays suitable for the analysis of the collected particles.

These three key elements have been addressed during the work on this thesis and will be discussed in more detail.

5.1.2 PEx formation

Exhaled breath contains very low amounts of non-volatiles. It is therefore essential to use a breathing manoeuvre that produces a high amount of particles so that a sufficiently large sample can be collected in as short time as possible. In addition, for reproducible sampling it should be known in what airway region the particles are formed. Thus, the use of a standardised breathing manoeuvre is vital when sampling particles from exhaled breath.

With the PExA instrument it is possible to sample particles using different breathing manoeuvres. The operator can use a valve to select specific exhalations or parts of an exhalation to sample. This offers a unique possibility to study the effect of different breathing manoeuvres on both the exhaled amount of particles and the composition of the particles. This knowledge is valuable when designing optimal breathing manoeuvre for future clinical studies.

Based on the previously existing knowledge and the new knowledge obtained during this study, the optimal breathing manoeuvre for sampling a high amount of PEx from small airways should maximise the number of closing and reopening airways. This can be achieved by exhaling to RV and holding the breath for a few seconds, followed by a fast inhalation to TLC and an immediate and moderately fast exhalation back to RV. Very slow exhalation flows should be avoided to minimize deposition of particles in the small airways. Deposition by gravitational settling is time dependent and Holmgren *et al.* found that 5 s breath holding at TLC could reduce the exhaled particle number concentrations by as much as 40 % (102). The present thesis also clearly showed the importance of breathing manoeuvre in respect to particle formation site. Very fast exhalation flows should also be avoided since this may generate particles in the proximal and upper airways. With the suggested breathing manoeuvre, both a high exhaled amount and an airway region specific sample can be obtained.

5.1.3 PEx sampling

Non-volatile substances, e.g., proteins and lipids, can only be transported in exhaled air as parts of a particle in the aerosol. Therefore, an efficient sampling method for non-volatiles in breath should focus on the collection of particles. Sampling of particles has the advantage that the particles constitute a micro sample of undiluted RTLF. Non-invasive methods are unlikely to induce alterations in the airway RTLF composition, in contrast to BAL and sputum sampling procedures that can induce an inflammatory response.

The major limitation with the non-invasive methods is the low amount of RTLF that is exhaled and that no cells can be sampled. It is therefore essential that as much as possible of the RTLF present in breath is collected. We clearly demonstrate a significantly higher efficiency of RTLF sampling when using the PExA method compare to the EBC method, evident by the reproducible analysis of SP-A in PEx but not in EBC samples of the same exhaled volumes of breath.

Contamination from other biological fluids is a problem often associated with other RTLF sampling techniques: blood contamination for BAL samples, and saliva contamination for sputum and EBC samples. If not controlled, such contamination might cause erroneous data interpretation. Due to the non-invasive nature of the PExA method we do not expect any blood contamination, which is strongly confirmed by the observed stable ratios of SP-A to albumin in PEx. The albumin concentrations in the PEx sample are not more the twice of the locally produced SP-A, which further supports the absence of blood contamination. The earlier proteomic study showed the absence of amylase in PEx samples, supporting absence of saliva contamination (104). This was further confirmed in a study by Willner *et al.* that found no codeine in PEx samples after subjects had rinsed their mouth with a codeine solution (manuscript in preparation). Based on these findings, we conclude that the PExA method is capable of providing a pure sample of RTLF, free of contamination from other biological fluids.

Filtration and impaction are two of the most commonly used methods to sample particles from an aerosol. Both of these methods result in a dry sample on a solid support. This is advantageous for sample storage since hydrolysis and proteolysis require presence of water. The disadvantage is that the sample often has to be quantitatively extracted into a specific solvent before analysis. This can be a limitation when several assays with different solvents are required. One solution is to split the sample in two equal parts on the membrane and split the membrane in two parts before extraction. This can be achieved by using different nozzle configurations above the impaction plate.

The sampling surface should have properties that make both the sampling and the extraction efficient. It is difficult to construct small and efficient filters for sampling the size range of particles found in exhaled breath. These filters often have a high flow resistance or a large volume. The sampling membranes used in the impactor are only 140 µm thick and 25 mm in diameter. This makes it possible to extract the particle sample from the sampling surface in a very low volume of solvent, which minimizes the dilution of the sample.

It is possible that particles are formed in a way that does not reflect the overall RTLF composition. The RTLF in the alveoli is composed of a surfactant layer and an aqueous fluid layers beneath. If particles are formed with a surface layer of surfactant lipids and an aqueous interior, then particle composition would be influenced by particle size as the volume to surface area ratio would depend on particle size. However, in practice the particle size distribution is generally very stable, and seem not to be altered in for example asthma, and the average effect on the collected sample appears to be low. Particles can grow and shrink

before they are measured by the OPC and this could result in an over or underestimation of the collected volume of RTLF. To minimize this effect, the PExA instrumentation is heated to a similar temperature to exhaled breath and the air in the instrument is saturated with water.

With the PExA method the samples can be collected and extracted with both high efficiency and high reproducibility

5.1.4 PEx analysis

Most methods for retrieving RTLF have unresolved problems with unknown and variable dilution. The volume or mass of the retrieved RTLF is either not known or not reproducible. To estimate the concentration of analytes in the RLTF it is essential to know how much RTLF that has been retrieved. The possibility to estimate the amount of collected material is a key objective with the PExA method.

The mass of sampled particles using the PExA method is generally less than 1 μg if the sampling time is less than 30 minutes, and methods for weighing such a sample are not readily available. For this reason, the PExA method uses an OPC for estimating the sampled particle mass. The ratio of collected analyte mass (determined by chemical analysis) to collected particle mass (calculated from particle concentrations and size) is expected to be constant regardless of the total PEx mass sampled. The linearity between the collected PEx mass and the measured mass of SP-A, albumin, DPPC and POPC strongly support our calculation of the collected particle mass. There is a probability of a systematic error, however, this should not affect the overall observed trends of the measured concentrations and can be corrected in the future.

The low amounts of collected material put strong demands on the selection of chemical methods used for PEx analysis. In this work, to satisfy the requirements of the individual sample analysis, we used ELISA for protein assays and SRM for lipid assays. Both techniques are known to be sensitive, however, a lot of samples are often found to be close to the detection limits. In general low amount of samples should be considered just a temporary limitation due to the constant development of new analytical techniques.

A limitation with the PExA method has been that samples from individuals exhaling a low amount of PEx often are close to the LoQ of the chemical analysis whereas samples from individuals that produce high PEx amounts have to be further diluted. This has been solved by calculating the sampled PEx mass online during the sampling and adjusting the sampling time based on the set value of sampled PEx mass. The approximate concentration of analytes in

PEx is usually known from previous measurements and it is easy to estimate the appropriate PEx mass to collect. When all samples contain the same PEx mass they are diluted to the same degree during extraction and are likely to be within the quantification range of the selected chemical assay.

However, because the actual mass is not validated comparisons between PEx concentrations and other sample types e.g. blood is problematic. If several analytes are measured in the sample, we can choose to work with the concentration ratios instead of their absolute concentration. These ratios will be independent of the sampled PEx mass, eliminating errors in PEx mass calculation. For example, the ratio of DPPC to POPC is similar in PEx samples and in surfactant prepared from BAL samples. Therefore, I believe that both ratios and wt% concentrations in PEx should be reported when possible.

5.1.5 Biomarkers

A biomarker should ideally be able to measure a relevant biological process or biological change on an individual level. This requires that the method for analysing the biomarker has a sufficiently high accuracy and reproducibility. A biomarker is more valuable and more easy to use in a clinical setting if there is a reference interval that identifies an abnormal value, indicative of a particular disease

Currently methods to measure SP-A, albumin, DPPC and POPC in RTLF collected with the PExA are available, with the first two having been developed during this thesis. All these biomarker candidates have functions that make them interesting to evaluate as biomarkers for airway inflammation. It is however currently not known how their concentrations would be changed by inflammation. Different types of inflammation and different inflammation degrees may also produce different responses.

In the present work we found that the PEx mass concentrations decreased after birch pollen exposure for individuals with asthma and birch pollen allergy. This indicates that also the amount of exhaled particles can be a biomarker. Particle formation and deposition are influenced by a number of biological processes that are related to inflammation e.g. accumulation of fluid and airway narrowing and are thus potential biomarkers for these processes. It is therefore recommended to also evaluate how the exhaled amount of particles can be associated with different lung diseases.

The methods to sample PEx and analyse SP-A and albumin, developed during the work described in this thesis, have been used with promising results in several clinical projects. Low SP-A concentration in PEx was associated with

COPD in a study by Larstad *et al.* (160). Studies on lung tissue have found that SP-A levels were lower in COPD patients than in non-smoking controls (161), indicating a dysfunctional SP-A production by type II cells. Low levels of SP-A in PEx may be a predictor of bronchiolitis obliterans syndrome after lung transplantation (162). Emilsson *et al.* found that SP-A and albumin concentration in PEx was lower in individuals with nocturnal gastroesophageal reflux, indicating that nocturnal gastroesophageal reflux affects the small airways (163).

It has become accepted that it is unlikely that a single biomarker can be used for diagnosis and treatment monitoring and that a panel of markers would be required to achieve this. Both ELISA and MS based techniques are capable for multiplex analyte assays, an attractive alternative for the samples of low quantities. Method development for simultaneous detection of several analytes is more complex, but might be a necessary step in further development of the PExA method.

5.1.6 Variability in exhaled particle amount

The reason for the large inter-individual variability in exhaled particle mass concentration is currently not known. One hypothesis that has been suggested is that airway closure and reopening occurs at different airway generations in different individuals. The length of each airway generation is very short in the periphery; the distance between airway generations 17 to 20 is less than 5 mm according to Weibel's lung model (6). If the airways branch by dichotomy the number of airways reopening could double for each airway generation. This would suggest a possible eight-fold increase in closing and reopening airways by a shift in location by only half a centimetre.

6 CONCLUSION

The work presented in this thesis has contributed to the development of a non-invasive method for sampling of RTLF with the purpose of studying airway inflammation.

PAPER I

The PExA method was more efficient than the EBC method for sampling of RTLF for subsequent SP-A and albumin analysis. The measured concentrations of SP-A and albumin should be adjusted for the variability in sampled PEx mass to improve reproducibility and to better reflect the RTLF composition. SP-A in blood does not correlate to SP-A in PEx.

PAPER II

In the group with mild asthma and birch pollen allergy, the exhaled PEx mass concentration was reduced after pollen exposure while no significant effect on SP-A or albumin concentrations in PEx was observed. In the studied group, exhaled PEx mass concentration may be a more sensitive measurement of airway inflammation than SP-A and albumin concentrations in PEx.

PAPER III

The concentrations of DPPC and POPC in PEx sampled with a forced exhalation were lower than in PEx sampled with the airway closure and reopening manoeuvre. This suggests that particles sampled with a maximal forced exhalation are likely from proximal or upper airways and contain very little surfactant. When a forced exhalation was used, the exhaled mass of PEx per litre of breath was 2.5 (95% CI 1.1–5.7) times that of the reference manoeuvre that used a slower exhalation flow.

PAPER IV

The calculated 90% reference interval was 1.9–5.3 wt% and 3.6–11.2 wt% for SP-A and albumin in PEx, respectively. SP-A concentration in PEx was not associated with age, gender, anthropometry, atopy or particle production in a healthy population. Albumin concentration in PEx was associated with exhaled PEx mass concentration, age and atopy and this has to be taken into account when designing studies and evaluating patient data.

7 FUTURE PERSPECTIVES

One of the major limitations with sampling particles is that the exact airway location where they form is not known. It is possible that formation of particles occurs at slightly different sites in different individuals even when the breathing manoeuvre is standardised. Currently it is not well known how the RTLF composition changes throughout the airways. This information may be used for understanding where particles are formed. Vice versa, knowledge of particle formation can also be used to study the RTLF composition in different airway regions. Future projects that could increase the potential of the PExA method are:

Multiple reaction monitoring (MRM) methods may be used to replace ELISA methods for protein analysis. SP-A has proven to be a problematic protein to analyse using ELISA. Mass spectrometric methods for quantifying SP-A is therefore an attractive alternative to the ligand binding assays. MRMs directly quantify the protein molecules, instead of quantifying protein interaction, which is an indirect measurement of the present molecules (164, 165). This project has been started by preparing purified SP-A, which is required during the method development.

Validating the PEx mass calculation from OPC data by an absolute method e.g. weighing the sample.

Fractionating the PEx sample into a fraction above and below mass median aerodynamic diameter and comparing the composition between the large and small particle fractions. This project has been started and first samples have been collected.

Aerosol generator, which generates an aerosol from a liquid with a known concentration of the analyte. This aerosol could be sampled with the PExA instrument and the estimated liquid concentration in PEx could be compared to the liquid with a known concentration. This would be an advantage during method development where collection of PEx samples is very time consuming part of the projects.

Sensitive multiplex assays can analyse many proteins in the same volume of solvent, eliminating the need to split the sample volume. This could open up new possibilities to measure large panels of markers and finding biomarker fingerprints for biological pathways.

ELISAs with PCR could improve the sensitivity of conventional ELISAs by amplifying the signal using the polymerase chain reaction.

Antibody coated sampling membranes could potentially be used to make an analysis directly on the membrane. PEx is already sampled as small spots on the membrane. If the membrane was coated with antibodies the analysis could be made directly on the membrane using extremely low solvent volumes, thus the dilution that takes place during extraction could be avoided.

8 GENDER ASPECTS

The study populations were relatively balanced between males and females in all studies. The research group that planed and conducted the research was balanced between males and females. Asthma is more frequent in women than in men over the age of 35 years but difference in prevalence of allergic asthma are small (166). No clear difference between men and women was observed in the studies.

9 ETHICAL CONSIDERATIONS

The PExA method samples particles from breath and is considered a safe and non-invasive method. No harmful effects have yet been observed. However, the effect of repeatedly inducing a high degree of airway closure and reopening is not fully known, therefore a small risk cannot be excluded.

10 DISCLOSURE STATEMENT

Per Larsson is a shareholder in PExA AB (www.pexa.se). PExA AB is selling an instrument similar to the PExA instrument used in the studies presented in the thesis.

ACKNOWLEDGEMENT

Samtliga arbeten i den här avhandlingen är ett resultat av det goda samarbete som finns i forskargruppen. Jag vill uttrycka min tacksamhet till samtliga medlemmar. Några som varit speciellt betydelsefulla för den här avhandlingen är:

Ekaterina Mirgorodskaya, att ha dig som huvudhandledare har varit en stor trygghet. Du är en förebild för hur en skicklig kemist och forskare ska vara.

Anna-Carin Olin, forskningsgruppsledare och bihandledare, du var den som gav mig chansen att påbörja resan till disputation. Tack för att du gett mig tid att fördjupa mig i forskningsfrågorna och för allt du lärt mig om forskning.

Mona Lärstad, bihandledare, tack för allt stöd på vägen.

Björn Bake diskussionerna vi har haft runt lungfysiologin tillhör det mest givande från doktorandtiden.

Evert Ljungström som designat PExA instrumentet och förklarat hur det fungerar.

Gunilla Runström som hjälpt mig med språket i manuset och bidragit i diskussioner kring de kemiska analyserna.

Ann-Charlotte Almstrand som introducerade mig till PExA instrumentet och visade hur en avhandling på PExA metoden ska skrivas.

Anna Bredberg för alla år vi var kollegor och för att jag fick använda dina fina bilder i avhandlingen.

Oscar Hammar för alla lunchtimmar du lagt på att förklara svåra statiska frågor på ett sådant sätt att till och med jag förstår.

Anita Wallin för att du körde lipidproverna och för alla bra diskussioner.

Marianne Andersson för alla intressanta diskussioner kring hur vi ska förbättra PExA metoderna och för all hjälp med insamling av prover.

Bo Strandberg för att du lärde mig analysmetoderna på laboratoriet. Du har varit en fantastik labbchef som styrt upp verksamheten med ditt kunnande.

Hatice Koca Akdeva som är vår expert på ELISA-analys och för att du analyserade proverna i delarbete IV.

Göran Ljungkvist som varit min kontorsgranne och därmed fått svara på frågor om både det ena och det andra.

Annika Claesson och Kristina Wass som hjälpt till med de kliniska studierna.

Helene Olofson (Holmgren) dina studier på PExA metoden har varit betydelsefulla for mitt arbete och tack för att jag fick låna dina bilder. Jag vill också tacka alla deltagare studierna, utan er hade det inte varit möjligt att göra några studier.

Jag vill också tacka mina föräldrar, **Katarina** och **Jan-Håkan,** för att ni trott på mig och uppmuntrat mig.

Framför allt vill jag tack **Meta** och **Algot**, min familj, jag ser fram emot att äntligen kunna spendera mer tid med er igen. Jag är otroligt tacksam för att jag har er och ni är viktigare än alla avhandlingar i världen.

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APPENDIX A

METHOD DEVELOPMENT AND VALIDATION

11.1Experiments before paper I

11.1.1 Addition of valve system

The 3.6 L breath reservoir could be too small to buffer all of the exhaled air when exhalations were made into the PExA instrument continuously. This problem was solved by installing larger reservoir (6 L) and a valve after the mouthpiece. With the valve installed it was possible to direct the exhaled air either to the PExA instrument for sampling or to ambient air, i.e. the individual could be disconnected from the sampling by the operator while continue breathing in the mouthpiece. The valve made it possible to sample only selected exhalations and to sample each exhalation fully before the sampling the subsequent exhalation.

11.1.2 Modifications to impactor

Particle deposition on at all stages of impactor were evaluated, by collecting and analysing exhaled material at all three stages. The particles were extracted and SP-A was measured by the in-house ELISA. In addition, the silicon diaphragm in the mouthpiece that is directly in the flow path of the exhalation flow was tested for SP-A content. The percentage of total SP-A recovered on each stage was the following: 72% for 0.5–2.0 μm stage, 22.6% for 2.0–7.0 μm stage, 4.1% for >7.0 μm stage. Only 1.3% of protein amount was detected on the silicone diaphragm.

To increase the fraction of sampled material, the impactor was modified to sample particles in 0.5–7.0 μm range on one impactor plate.

11.1.3 Extraction of PEx for ELISA

SP-A extraction from PEx was compared between 0.05% tween, 20 mM nOGP and 2 M urea. Tween generated the highest concentration of SP-A in extracted sampled and was selected for further experiments.

Tween concentrations between 0–0.2% (v/v) and the effect of sample ultra-sonication was tested on the standard serum. Only minor differences on the SP-A results were observed.

Spike and recover from the silicon surface was tested using the reference serum with known SP-A concentration. In an attempt to make the serum matrix more similar to the exhaled particle matrix, different concentrations of Curosurf (Nycomed, GmbH, Zurich, Switzerland) surfactant were added to the serum samples before spiking onto the silicon wafer. Tested Curosurf concentrations were: 0.0, 0.8 and 8.0 $mg \cdot mL^{-1}$. The samples were extracted by ultra-sonication for five minutes. No large effect of different Curosurf concentrations was observed and the recovered mass of SP-A was around 80–90% for all samples. The recovery of dried down serum standard was tested with recoveries of >90% after reconstituting the dried down samples.

SP-A extraction with tween using ultra-sound and subsequent sample concentration by vacuum evaporation was found appropriate for use in study I. The final protocol used in paper I is described in the method section.

11.1.4 Pre-study validation of in-house ELISA

The reportable range for the in house SP-A ELISA was estimated by running quadruplicates of the SP-A standard and plotting a precision profiles of %CV and %RE. The limit if quantification was determined to be 0.2 based on the performance profiles (fig 22).

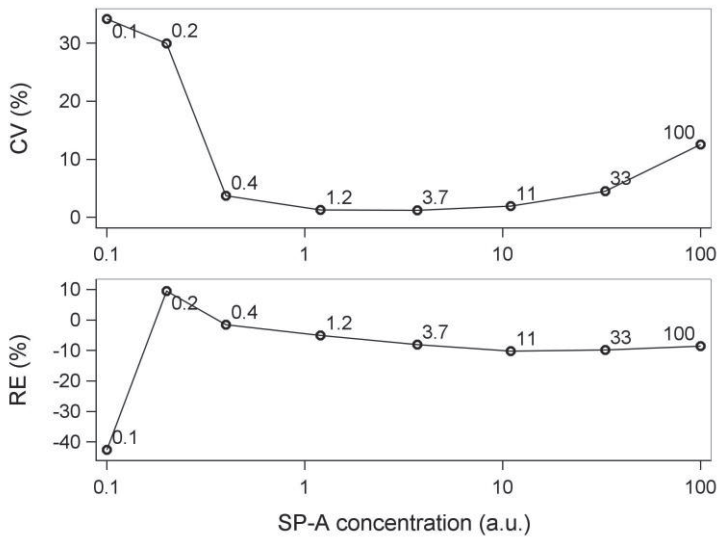


Figure 22. Performance profiles for standards on SP-A in-house ELISA. x-axis is on a decadic log scale. Data points are labelled with the concentration level. CV (%): coefficient of variation; RE (%): relative error; au: arbitrary units

The effect of freezing SP-A samples was tested by spiking and recovery using the serum standard. Two silicon wafers were spiked with 5 au of SP-A and the recovered amounts were 4.64 and 4.66, corresponding to a 93% recovery.

The repeatability of the assay was tested from a very large sample of pooled PEx (table 6). The pooled PEx sample was collected from six individuals and was sampled from 1000 L breath. The pooled sample was extracted and split into eight aliquots and run in quadruplicates on two ELISA plates.

Table 6. In-house SP-A repeatability

	Replicate			
Assay	1	2	3	4
Plate 1	1.6	1.5	1.6	1.6
Plate 2	2.4	2.6	2.4	2.5
Concentration in sample, arbitrary units				

The within and between assay CV was calculated using a One-way ANOVA. CV within assay was 3.9% and CV between assays was 31.5%. The reported inter-assay CVs in paper I was slightly different as it was calculated from the means of plate 1 and plate 2. Short term storage stability was tested on two PEx samples by extracting and analysing half the sample immediately and storing the other half in -20 C freezer for eight months before extraction and analysis. The recovery (unfrozen/frozen *100) were 86% and 93%. Using the same samples albumin was analysed in quadruplicates and the intra assay CV was estimated to 4.8% (72.1, 68.9, 75.3, 76.9 $ng \cdot mL^{-1}$).

SP-A was stable in -20 °C freezer for at least eight months. The in-house ELISA had acceptable performance in the range of 0.2–100 au. Large difference between assays was observed when a new batch of anti-bodies were used and this should be avoided within a study.

11.2 Experiments before paper II

11.2.1 Selection of PEx sampling surface

Six different commercially available membranes were obtained from Millipore (Billerica, MA, USA) and are listed in table 7. The silicon wafer that was prepared in house was used as a reference surface to ensure that the new sampling surface had a similar sampling efficiency.

Table 7. Sampling surfaces properties

	Sampling surface	Pore size	Thickness	Softness	User comment
1	Hydrophilic Polytetrafluoroethylene (FHLC02500)	0.5 µm	140 µm	High	Compliant
2	Hydrophilic Glass Fiber (APFC02500)	1.2 µm	240 µm	Medium	Porus
3	Hydrophilic Mixed cellulose esters (HAWP02500)	0.45 µm	150 µm	Low	Fragile
4	Hydrophilic Polytetrafluoroethylene (JHWP02500)	0.45 µm	65 µm	High	Compliant
5	Hydrophobic Polyvinylidene Fluoride (HVHP02500)	0.45 µm	125 µm	High	
6	Hydrophilic Polycarbonate (HTTP02500)	0.4 µm	10 µm	Low	Etched pores
7	Hydrophilic Silicon wafer			Crystalline	

Four volunteers from our department were recruited for the comparison of sampling surfaces. Each of the volunteers exhaled 60 litres of air on each of the seven surfaces in a randomized order. For surface six only three samples were obtained since only three membranes of this type were available at the time of the study. The samples were extracted from the silicon wafer by ultra-sonication for five minutes in 300 µL of a desorption solvent consisting of; 100 µL 0.01 M PBS/ 0.15M NaCl/ 0.13% Tween-20 (v/v)/ 1% BSA (w/v) and diluted with 200 µL water. From the silicon wafer the extraction was made in a glass vial and after ultra-sonication the sample liquid was transferred to a 1.5 ml polypropylene tube. From the membrane surfaces the extraction took place in Millipore ultra-free centrifugal tubes UFC30LH25 (Millipore, Billerica, MA, USA). Adsorbed liquid on the membranes were spun down using the filter insert and centrifuging at 12000 * RCF to recover all liquid in the vials. All

samples were evaporated to dryness using vacuum centrifugation and samples were stored dry at -20°C prior to analysis. For the analysis, the dry samples were reconstituted by adding 200 µL MilliQ-water for a final solvent concentration of 5 mM PBS/ 80 mM NaCl/ 0.06% Tween-20/ 0.5% BSA. Albumin amount was determined with a commercial ELISA kit (E-80AL, Immunology Consultant Laboratory, Newberg, OR, USA) using the protocol suggested by the manufacturer. All samples were analysed in duplicates and the mean concentrations were reported. Weight percent albumin in PEx was calculated and used as an estimate of the sampling surface performance. The results are presented in fig 23.

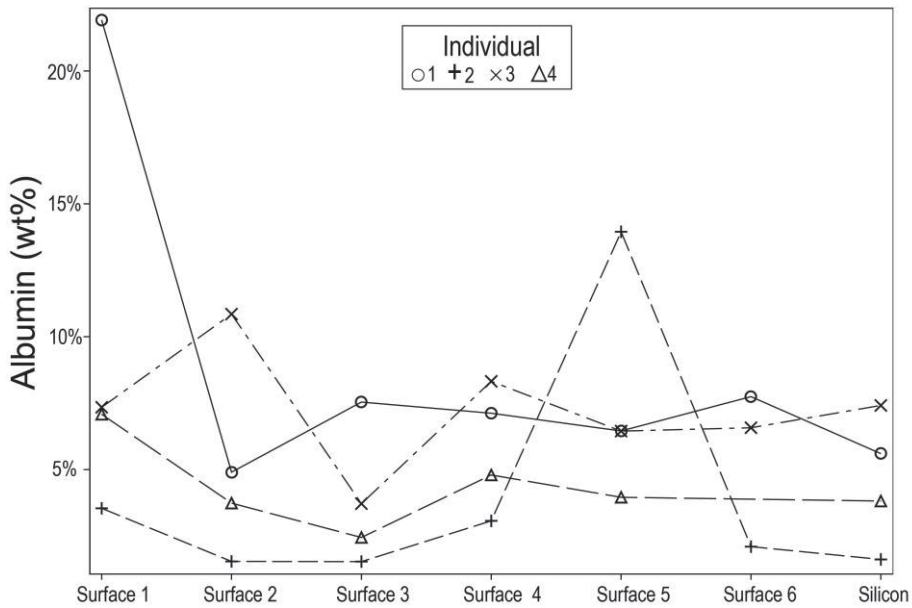


Figure 23. Albumin wt% in PEx sampled with different sampling surfaces.

The hydrophilic PTFE filters (filter 1 and filter 4) were easiest to fit on the impactor stage before sampling. They were also the easiest to cut out and fit into micro tubes. It was possible to recover almost all extraction solvent from the hydrophilic PTFE filters and they did not appear to release any contaminants to the samples. Surface 1 (FHLC02500) was selected as the sampling surface to replace the silicon wafers although surface 4 (JHWP02500) demonstrated very similar performance.

The membrane offers several practical benefits compared to the silicon wafer, they are delivered ready to use, they are widely available and the softness and

flexibility makes it possible extract the sample in low volumes of solvent in a polypropylene micro tube.

11.2.2 PTFE-membrane and electret filters compared

During a collaboration with Olof Beck at Karolinska Institute it was observed that more methadone could be sampled from breath using the SensaBues® method than the PExA method. Therefore, the filter used in the SensaBues device was tested as a sampling surface on the impaction plate in the PExA instrument. Breath samples were collected on all three levels in the impactor and the sampled amount of albumin from the PTFE-membrane and the sampled amount by the electret filter was compared. Four samples of 200 ng of PEx were collected for each of the filter types and the results are presented in fig 24. The recovery of filters spiked with albumin standard was also tested to verify that the extraction efficiency was similar for both sampling surfaces. Spiked and recovery of albumin was close to 100% for both filter types.

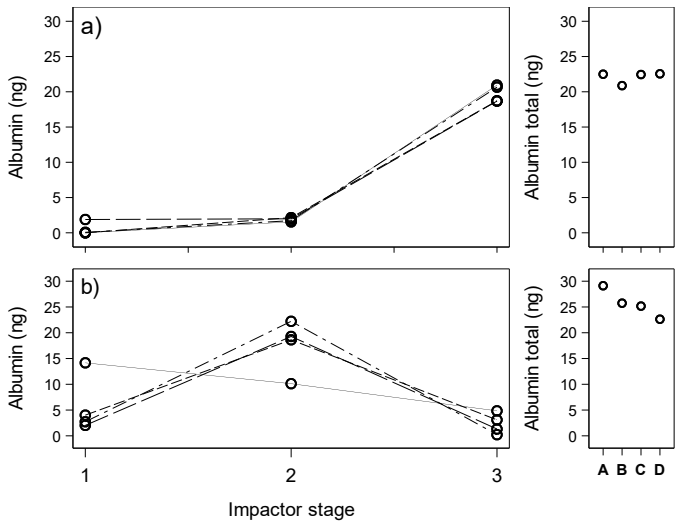


Figure 24. Sampling of albumin on three impactor stages compared. Four samples labelled A, B, C and D were collected for each of the sampling surfaces. a) PTFE-filters a) and b) electret filters. On the left the sampled albumin mass for each of the impactor stages are shown and on the right the total mass of sampled albumin is shown. Impactor stage 1 samples particles from 0.5–2.0 μm; Impactor stage 2 sample particles from 2.0–7.0 μm; Impactor stage 3 samples particles >7.0 μm.

The total amount of albumin, when the mass for each stage was summed, was very similar for both sampling surfaces and around 20 ng. The electret filter

was voluminous and porous and likely sampled particles by both impaction and filtration in impactor stage 2 whereas the PTFE-membrane is very thin and likely samples particles mainly by impaction on impaction stage 3.

A likely explanation for the larger amount of methadone obtained with the SensaBues® device is that it samples particles close to the mouth and collects particles outside of the 0.5–7.0 μm in diameter range that are sampled with the PExA instrument.

The PTFE-membrane has several properties that makes it better suited to be used with the impactor compared to the electret filter. A much lower volume of extraction solvent was required for extraction from the much smaller PTFE-membrane compared to the voluminous electret filter and it was easier to recover all extraction solvent from the PTFE-membrane.

11.2.3 External and internal validity

The external validity for the PExA method to measure wt% albumin in PEx was evaluated by sampling PEx from healthy individuals at different research centres (Umeå, Leicester, Gothenburg). From each individual samples were collected from an increasing volume of exhaled breath to evaluate if the association between sampled PEx mass and albumin mass was similar between centres (fig 25).

In gothenburg two samples (Gothenburg 1 and 2) were collected based on a predetermined PEx mass (165 ng). A nozzle pattern in the impactor that split the PEx sample in two halves on the sampling membrane was used. The membrane was cut in half and analysed separately. Both halves of the sampling membranes generated similar results (fig 25).

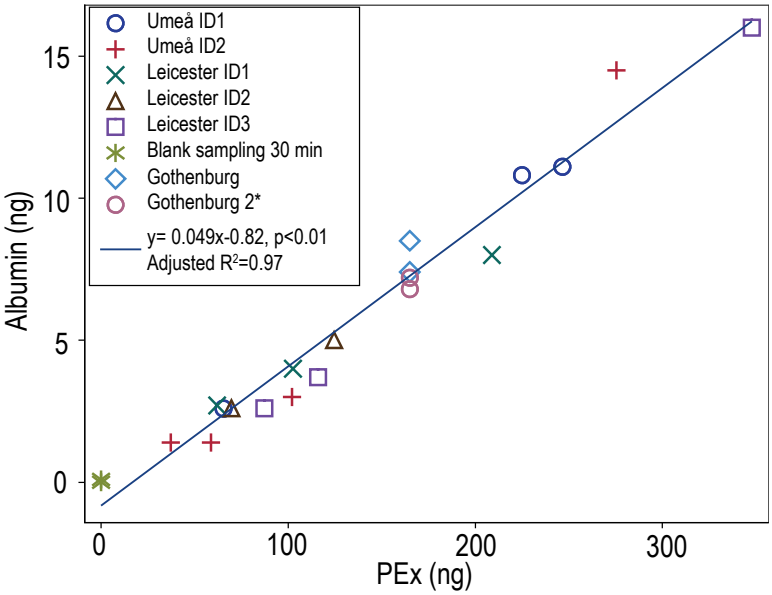


Figure 25. Association between sampled albumin mass to sampled PEx mass. The samples are labelled by the sampling location and by the id of the individuals breathing. In Gothenburg a predetermined PEx mass of 165 ng was sampled.

11.2.4 Particle measurement and volume measurement validation

Particle measurement

The within and between variability between different Grimm OPCs was evaluated by measuring on a stable aerosol of monodisperse latex spheres generated by a particle generator from TSI (fig 26). The Aerosol was dried by running the aerosol through tube filled with silica gel so that only the dry latex spheres were measured. The within instrument and between instrument CVs were calculated by one-way ANOVA (table 8). To make the dataset balanced data from the 250 first observations were used.

Table 8. Grimm OPCs compared

	Concentration (nr/L)	Concentration (ng/L)
Repeatability	2.01%	2.50%
Intermediate precision CV	7.7%	13.6%

Because mono disperse particles was used the particle mass concentration can appear to have a larger between instrument variability than what is expected when measuring on polydisperse spheres. If the size of the particle is close to the size bin boarders different instrument may classify the particle do either the larger or the smaller bin. If the aerosol had a wide range of particle sizes this effect at the size bin limits would influence the results to a lesser degree.

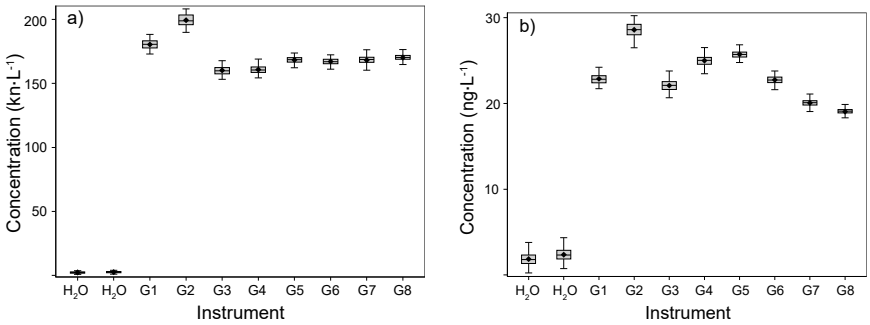


Figure 26. Grimm optical particle counters compared.

Volume measurement

The accuracy of the volume measurement was tested on three instruments using a 3 L calibration syringe (table 9). The flow was measured by a flow meter installed between the syringe and the PExA instrument and the flow was also recorded by the internal flow meter. The 3 L of air was injected using different flow rates between 1000 mL·s^{-1} and 3000 mL·s^{-1} to confirm that the volume measurements were not influenced by flow rate. Instrument PEx3 and PEx8 was of the same design whereas PExA2.0 was an upgraded version developed by PExA® AB, this instrument used a different kind of flowmeter for the internal measurements (SFM3100-VC, Sensirion, Stäfa, Switzerland) that measures flow based on the concept of convective heat transfer. From the results it could be observed that the PEx3 instrument had a slightly lower internal volume measurement by around 10% this remained the regardless of flow meter used. This was caused by a small leakage in the breath reservoir that had to be sealed.

Table 9. Internal external volume measurements

Instrument	Internal (mL) Mean (95%CI)	External (mL) Mean (95%CI)
PExA2.0	2950 (2868–3033)	2831 (2786–2877)
PEx3	2563 (2510–2615)	2867 (2849–2884)
PEx8	2772 (2748–2796)	2734 (2685–2783)

11.2.5 Comparison of SP-A ELISA

To overcome the problem of lacking a pure protein standard a commercial SP-A assay from Biovendor (RD191139200R, Brno, Czech Republic) was evaluated. The in house ELISA was compared to the Biovendor ELISA by running the same BAL samples on both assays and comparing the results (fig 27).

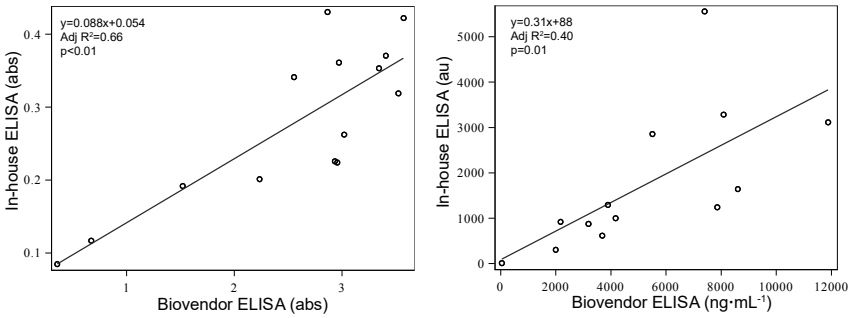


Figure 27. Association between BAL samples measured with in-house ELISA and Biovendor ELISA. In addition to the BAL samples a control sample supplied with the Biovendor kit (38 ng·mL^{-1}) was included in both assays.

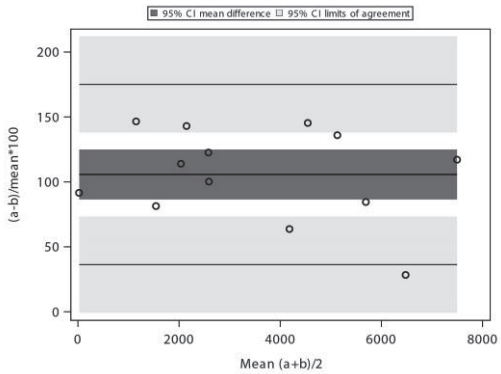


Figure 28. Bland Altman plot of the percent difference between method a and method b. a: SP-A concentration measured by the Biovendor ELISA (ng·mL^{-1}); b: SP-A concentration measured by the in-house ELISA (au).

Comparing the assays was not straight forward since the in-house ELISA did not have a pure SP-A standard and the amount of standard supplied by Biovendor was insufficient to be used for both assays. The assays are compared by evaluating scatter plots with correlation and by Bland Altman plots (fig 28). The coefficient of determination was quite low, indicating that the agreement

between assays was rather poor. From the regression coefficient it can be seen that 1 au translated to roughly 3 $ng \cdot mL^{-1}$ on the Biovendor assay. An arbitrary unit corresponding to 3 $ng \cdot mL^{-1}$ suggests that the pooled serum used as a standard had a concentration of approximately 900 $ng \cdot mL^{-1}$. When the serum used as reference for the in-house assay was analysed on the Biovendor assay the concentration was only around 15 $ng \cdot mL^{-1}$ and a good linearity with dilution was observed. Biovendor reports a normal range of SP-A concentrations in serum for their SP-A assay of 13–65 $ng \cdot mL^{-1}$, determined from measurements on 40 healthy volunteers. This huge difference in observed concentrations for the reference serum is a cause for concern and no clear explanation have been found. It may be that the two assays recognise different forms of SP-A e.g. monomers vs multimers. Cross-reactivity and heterophilic antibodies are a common sources of error that can increase signal intensity. A heterophilic antibody can crosslink the capture antibody with the detection antibody and create a false signal as illustrated in fig 29. The risk of this occurring increase with the number of different antibodies in the sample and with their concentration. The serum used as reference was pooled from several subjects that were selected because they generated a high signal. This increase the risk of having antibodies that can interfere with the ELISA assay.

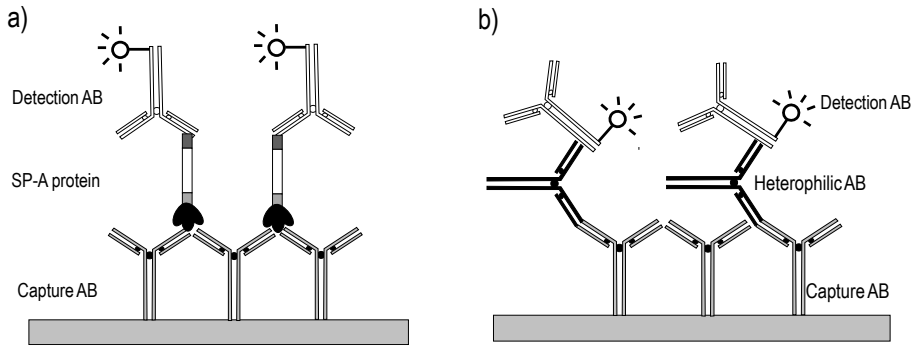


Figure 29. Heterophilic antibodies may cross link the detection antibody to the capture antibody and generate a false signal. a) The desired antibody SP-A binding and b) the heterophilic bindning creating a false positive signal.

In PEx samples the risk of having interfering antibodies us much lower than in serum since it is a less complex sample with a much lower concentration of antibodies. While it may be difficult to estimate SP-A concentrations from the in-house ELISA the results are still semi-quantitative and the presence of heterophilic antibodies in the reference serum does not change the conclusions that were made regarding PEx in paper I.

The relatively poor agreement in the BAL-samples absorbance between the in-house assay and the Biovendor assay may be a result of the sub-optimal dilution and sample preparation. We have later observed that results at the upper concentration range of the assay are less reliable and samples should be diluted to the lower end of the calibration curve. By running samples at the lower end of the standard curve and solubilising SP-A in a thermo-mixer at 37 °C the reproducibility of the Biovendor assay was improved considerably.

11.2.6 Validation of Biovendor ELISA

Biovendor SP-A ELISA was developed for serum and BAL samples so pre-validation of assay performance on PEx samples was necessary. Assay performance was evaluated based on linearity of dilution assessment (table 10) and spiking-and-recovery (table 11). Initially the idea was to use the extraction buffer also as the diluent for the ELISA assays and only dilute samples to the minimum volume required. The extracted buffer used for sample extraction was 10 mM PBS pH 7.4/ 0.15 M NaCl/ 0.05% Tween-20 (v/v) and 1% BSA (w/v). The linearity with dilution was tested with extraction buffer used as diluent for a number of different sample types. Also included were BAL, serum and purified SP-A. Purified SP-A was kindly provided by Ida Tornoe and her colleagues at the Department of Immunology and Microbiology, Institute of Medical Biology, University of Southern Denmark, Odense, Denmark. BAL samples was kindly provided by Gert Riese at Department of Internal Medicine, Sahlgrenska academy at University of Gothenburg.

Table 10. Biovendor SP-A linearity with dilution

Sample	Dilution factor	OBS ng·mL^{-1}	EXP ng·mL^{-1}	OBS/EXP
PEx	1	10.8	NA	NA
PEx	2	5.7	5.4	105%
PEX	4	3.6	2.7	134%
BAL	100	3.0	NA	NA
BAL	200	1.1	1.5	75%
Serum	4	2.2	NA	NA
Serum	6	1.1	1.5	77%
Pure	10	45.6	NA	NA
Pure	20	22.5	22.8	99%
Pure	30	16.8	15.2	111%

Recovery was evaluated by preparing a PEx sample large enough that it could be split in three and still be within range of the calibration curve. For this

425 ng of PEx was sampled and extracted in 300 µL of 10 mM PBS pH 7.4/ 0.15M NaCl/ 0.05% Tween-20 (v/v) and 1% BSA (w/v), it was split into three equivalent samples. The three samples were spiked with SP-A standard to give an increase of 0-10-20 $ng \cdot mL^{-1}$. Recovery was also measured in BAL and serum by adding SPA to give an increase of 0-10-20 $ng \cdot mL^{-1}$.

Table 11. Biovendor SP-A spike-and-recovery

Sample	OBS $ng \cdot mL^{-1}$	EXP $ng \cdot mL^{-1}$	OBS/EXP
PEX_0	9.6	NA	NA
PEX_10	21.7	19.6	111%
PEX_20	25.1	29.6	85%
BAL_0	1.1	NA	NA
BAL_10	9.4	11.1	84%
BAL_20	16.4	21.1	78%
SERUM_0	0.5	NA	NA
SERUM_10	4.6	10.5	43%
SERUM_20	7.9	20.5	39%

The poor linearity with dilution and poor recovery of spiked samples for serum may be a result of the buffer that did not contain Ca^{2+}. Biovendor reports that EDTA serum has 85% reduction in measured concentrations. This suggests that the antibody SP-A interaction is Ca^{2+} dependent, EDTA chelates Ca^{2+} and this reduces SP-A binding to antibody in these sampled. When serum samples were diluted with the Biovendor diluent the measured concentrations increase and a much better linearity with dilution was observed. When assay diluent was used, serum dilutions of 4, 8, 16 gave dilution adjusted values of 33.9, 34.4 and 34.4 $ng \cdot mL^{-1}$. This suggests that the assay diluent contained some component, likely Ca^{2+}, that increased that amount of detected SP-A in serum. The measured concentration of the purified SP-A was close to the concentration reported by Ida Tornoe and her colleagues, our results were around 90% of the expected value even though the sample had been stored for a couple of years in a -20 freezer. This was an indication of an acceptable assay precision.

11.2.7 Optimization of sample extraction

Different protocols for extraction of SP-A and albumin in PEx sampled on PTFE-membranes were tested. Albumin ELISA was purchased from ICL (E-80AL, Immunology Consultant Laboratory, Newberg, OR, USA). The parameters ultra-sonication, temperature and tween concentration were tested

(table 12). All extraction took place in a thermomixer (Eppendorf thermomixer comfort, Hamburg, Germany) for 60 minutes at 400 rpm.

Table 12. Test of different extraction conditions for extracting SP-A and albumin from PTFE-membranes

Different extraction conditions	Tween %* (v/v)	Temp* (°C)	Ultra-sound* (min)
Condition 1	0.05	40	5
Condition 2	0.10	40	5
Condition 3	0.05	24	5
Condition 4	0.05	40	no
* 10 mM PBS and 1% BSA (w/v)			

Each extraction condition was tested on three PEx samples. From each sampling membrane the extraction was repeated three times to measure the decline in extracted amount between successive extractions. The idea was that the extracted amount should be very low on the second and third extraction if the first extraction was efficient.

Table 13. Extracted mass of SP-A from PTFE-membrane

Condition	Sample	First (ng)	Second (ng)	Third (ng)	Second/First
1	1	7.2	1.5	1.1	21%
1	2	7.6	2.1	1.1	27%
1	3	7.9	1.4	0.4	17%
2	1	7.4	0.6	0.2	8%
2	2	25.0	2.9	0.7	12%
2	3	17.4	0.9	0.1	5%
3	1	2.1	1.7	1.9	80%
3	2	7.3	0.8	0.6	11%
3	3	13.4	7.6	22.8	57%
4	1	6.4	1.5	0.6	23%
4	2	4.8	1.1	0.5	23%
4	3	4.3	1.1	0.2	24%
Median (Q1–Q3)		7.4 (5.6–10.7)	1.4 (1.45–1.9)	0.6 (0.3–1.1)	22% (12–26)
The four different extraction conditions are presented in table 11. For each sample the extraction procedure was repeated three times and the extract was analysed for SP-A and albumin.					

The results for SP-A extraction are shown in table 13. It appears that the extraction protocol that used a lower extraction temperature of 24 °C resulted in a lower proportion of extracted SP-A. Around 95% of alveolar SP-A is bound to the surfactant lipids it is likely that the lipids take on a more liquid state at higher temperatures and then become more extractable. Increasing tween concentration may also increase the extracted fraction slightly. The effect of ultra-sonication appeared to be small. Albumin extraction was close to 100% for all tested protocols.

11.2.8 Modifications to ELISA protocols

Slightly different protocols were used in the papers and they were gradually modified based on experience. ELISA protocols suggested by the manufacturers have been modified for better reproducibility.

Currently suggested SP-A protocol:

- the standards are prepared in a modified diluent prepared by mixing extraction buffer and supplied SP-A assay diluent in the proportions 1:2 to match the solvent of our samples.
- the incubation between protein and antibody is made on a plate shaker at 300 rpm for 2h at 37 °C. Recommended by the manufacturer is 2h at 37 °C without shaking.
- the range for the SP-A ELISA is reduced as concentrations in the upper range of the assay were observed to have a lower reproducibility.

Currently suggested albumin protocol:

- the standards are prepared in a modified diluent prepared by mixing extraction buffer and supplied albumin assay diluent in the proportions 1:2 to match the solvent of our samples
- the incubation between protein and antibody is made on a plate shaker at 300 rpm for 1h at 23 °C. Recommended by the manufacturer is 1h at 22 °C without shaking.

11.2.9 Performance of Biovendor SP-A ELISA

The repeatability of the full method, sampling, extraction and analysis was evaluated by sampling 120 ng of PEx seven times from one subject. The results are presented in table 14. The assay range was also evaluated by plotting the CV and RE for repeated measurements on the standard samples (fig 30).

Table 14. Repeated measurements of SP-A in PEx sampled on PTFE-membranes

Sample	Well 1	Well 2	Average	CV
PEX1	31.5	29.3	30.4	5.0%
PEX2	24.0	33.9	28.9	24.0%
PEx3	31.9	63.7	47.8	47.0%
PEx4	32.4	51.6	42.0	32.0%
PEx5	33.9	31.1	32.5	6.0%
PEX6	27.4	26.7	27.0	2.0%
PEx7	30.7	26.0	28.4	12.0%
120 ng of collected PEx and analysed in duplicates.				

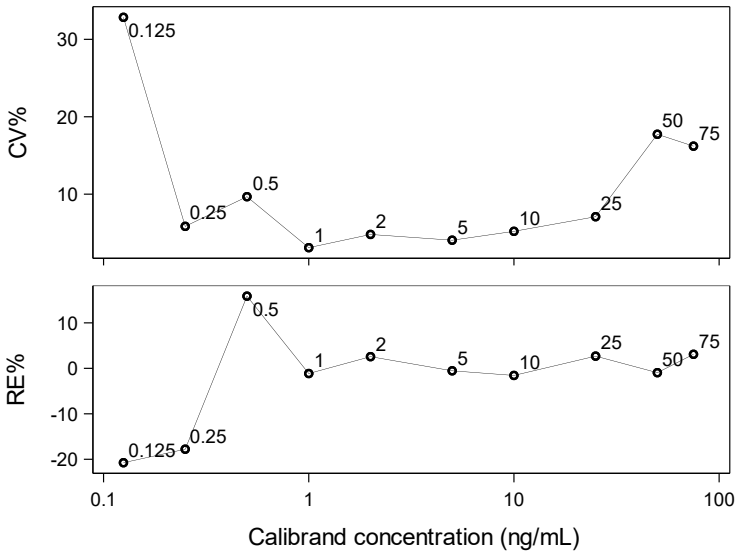


Figure 30. Biovendor SP-A assay performance of calibrands.

11.3 Experiments before paper III

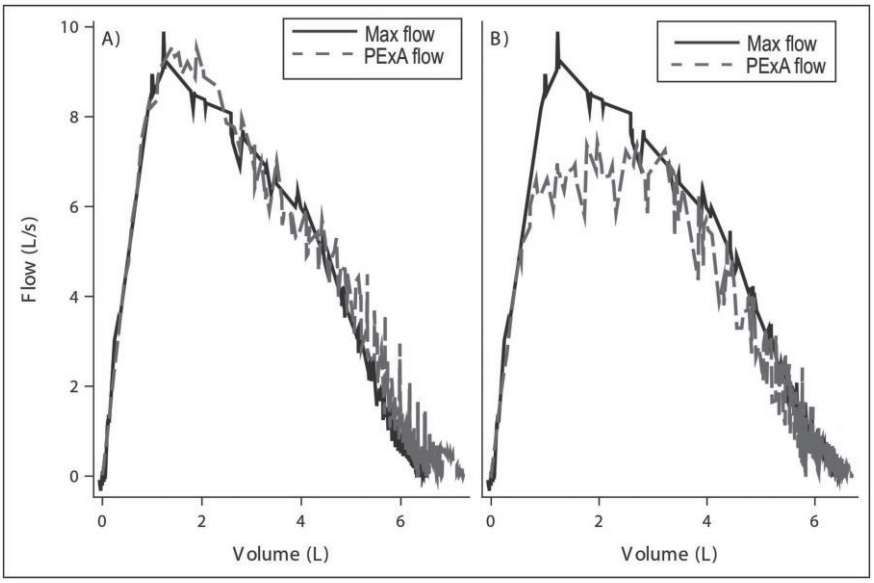
For paper III a new study protocol that address possible weaknesses in the first protocol was evaluated.

The purpose of the experiments was:

1. Ascertain that maximal expiratory flow into the PExA instrument is not reduced by the flow resistance of the instrument
2. Ascertain that particle losses in mouthpiece are low
3. Find a biological marker that is characteristic of peripheral airways that can be measured in as little as 10 ng of PEx

11.3.1 Maximal expiratory flow

The main source of flow resistance was found to be the Spiral-Type diaphragms at the inhalation and exhalation ports in the mouthpiece. A



maximal exhalation flow volume curve of one subject with a relatively high peak flow was compared with and without the 1420-A non-rebreathing valves connected to the mouthpiece fig 31.

Figure 31. A) Flow volume curves with PExA instrument (grey dotted line) was compared to maximal flow volume curves measured by exhaling through only a spirometer (black solid line). B) Flow volume curves with PExA instrument including 1420-A non-rebreathing valve (grey dotted line) was compared to maximal flow volume curves (black solid line). The curves are mean values from ten exhalations of each type. The PExA instrument has very little influence on the flow volume curves but when the 1420-A valve is installed the peak flows are reduced.

11.3.2 Particle losses in mouthpiece

The exhaled particle concentrations were recorded during the measurement and the number of particles >2.36 μm was clearly lower when the non-rebreathing valve was installed fig 32.

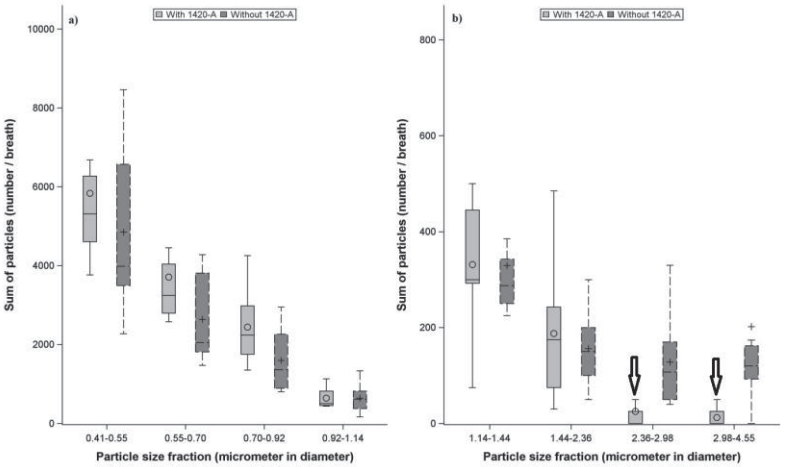


Figure 32. Particle losses in mouthpiece and valve during forced exhalations into the PExA instrument. Box plots of number of particles per breath with and without the 1420-A valve from 10 forced exhalations. Horizontal line, median; box, first and third quartile; whiskers, range excluding outliers, symbols in box represents the mean and symbols outside of the box represent outliers. a) Particles from 0.41–1.14μm. b) Particles from 1.14–4.55 μm. Particles losses in the 1420-A valve can be seen for particles larger than 2.36 μm. The particle size fractions where there were significant changes are indicated by arrows.

11.3.3 Changes to the study protocol

1. The 1420-A non-re-breathing valve was removed and exhalations that were sampled and measured were made directly into the PExA instrument using only a small open mouthpiece connected to the instrument tubing.
2. Instead of analysing SP-A, which was difficult to measure in samples with less than 20 ng of PEx, the surfactant lipid DPPC was measured.
3. The flow rates of the non-forced exhalation in the pilot study were kept very low to avoid flow induced particle formation. Because flow induced particle formation was not as large as we expected the exhalation flow of non-forced exhalations was increased from 0.4 $L \cdot s^{-1}$ to 0.8 $L \cdot s^{-1}$ to reduce the time difference it takes to perform the manoeuvres. Deposition of particles during the manoeuvres is time

dependent and the time difference between manoeuvres should be kept as short as possible.

11.4 Impactor modifications

Equation for calculating the stokes number

$$Stk_{50} = \frac{\rho_0 * U_0 * d_{50_0}^2 * C_{c0}}{18 * \mathrm{h} * 0.5D_j}$$

Stk_{50}= From the stokes number it can be calculated at what particle size the collection efficiency of the impactor stage is 50%.

ρ_0=density of particle

U_0 =Velocity of gas when exiting the nozzle

d_{50}=Particle diameter for 50% collection efficiency

C_c=Cunningham correction factor for small particles that can slip on gas molecules instead of pushing them.

h=Viscosity of gas

$0.5D_j$= Nozzle or jet radius of the impactor

The Decati impactor is specified for a flow rate of 150 mL/s (for sampling of particles PM1, PM2.5 and PM10. In the PExA instrument it is modified by increasing flow rate 1.5 times and the jet velocity of the PM1 stage is increased by 2 by blocking 10 of the 20 jet nozzles. A new cut off is calculated by using the new parameters and the stokes equation. The properties of the impactor are not changed and are the same before and after the modifications. A new d_{50} is calculated from the stokes equation exchanging the values and using the Cc for smaller particles with a higher slip factor 1.4 compared to the larger particles 1.2.

$Stk_{50(new)}$=$Stk_{50(old)}$

$$\frac{\rho_0 * 1.5 * 2 * U_0 * d_{50_{new}}^2 * 1.4}{18 * \mathrm{h} * 0.5D_j} = \frac{\rho_0 * U_0 * d_{50_{old}}^2 * 1.2}{18 * \mathrm{h} * 0.5D_j}$$

$$1.5 * 2 * d_{50_{new}}^2 * 1.4 = d_{50_{old}}^2 * 1.2$$

d_{50old}=1.0μm

d_{50new}=0.5345μm

Calculating the diameter for particles of exhaled liquid having a density if 1062 kg/m^3 from the aerodynamic diameter 0.5345μm

d_{50a}=d50*(ρ/ρ_{ref})$^{0.5}$

0.534=d_{50}*(1063/1000)$^{0.5}$ μm=0.519 μm rounded to 0.5 μm

In the smallest size bin 0.41–0.55 μm not all particles are sampled when the cut off is 0.5 μm.

The fraction of particles in this bin that are larger than the cut off is calculated by:

(0.55–0.50) fraction above cut off divided by the width of the bin (0.41–0.55) = 0.36=36% of particles are above the cut-off. This is an approximation some large particles pass and some small particles are sampled. A flat size distribution was assumed, look at the collection efficiency plot.

Because we only use one sampling level with a cut off of 0.5 μm we collect all particles on this level. This lead to the conclusion that for the smallest size bin we sample around 36% for particles between 0.55–4.55 μm we sample 100%.

The effect of particle growth and shrinkage is not adjusted for in this template. It is assumed that the supplied air is humidifier saturated with water at 36 C. Although the effect of water evaporation and condensation can be large it is difficult to predict and adjust.

/END